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IN VITRO GENOTOXICITY TESTS: CURRENT STATE OF AFFAIRS

Alberta
WORKERS' HEALTH, SAFETY
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IN VITRO GENOTOXICITY TESTS:

CURRENT STATE OF AFFAIRS

Prepared by:

Brenda Weir, B.Sc.(Hons), M.Sc.

For:

Occupational Hygiene Branch
Occupational Health and Safety Division
Alberta Workers' Health, Safety and Compensation

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With the increasing use of chemicals at work sites, has come a need for better understanding of the effects on the health of workers exposed to them. This study evaluates the use of short-term *in vitro* genotoxicity tests for assessing the carcinogenic potential of industrial chemicals.

Comments on the report and requests for further copies should be forwarded to this Branch.

David E. Gibson
Director
Occupational Hygiene Branch

Occupational Health and Safety Division
Alberta Workers' Health, Safety and Compensation
10709 - Jasper Avenue
Edmonton, Alberta
T5J 3N3
Telephone: (403) 427-2687

The views expressed in this report do not necessarily represent those of the Occupational Health and Safety Division, Alberta Workers' Health, Safety and Compensation.

ABSTRACT

This study was conducted to evaluate the current status of short-term *in vitro* genotoxicity tests, because results from these tests may be used by government agencies in assessing the carcinogenic potential of industrial chemicals or by-products of various chemical processes.

It has been well documented that many chemical mutagens are also carcinogens and in recent years the whole field of short-term mutagenicity testing has undergone rapid development. A great deal of interest has centred on bacterial mutagenicity studies and in particular the Ames assay. However, batteries of short-term tests provide a more accurate evaluation of the carcinogenic potential of a chemical than one single test, because a wider range of genetic end points, induced by different chemical classes, may be detected. Each group of tests has its own advantages and limitations and these are discussed individually. Generally, practical assay systems for application in screening programmes need to be sensitive, relevant and both time- and cost-efficient.

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TABLE OF CONTENTS

		Page
1.	PURPOSE AND OBJECTIVES	1
2.	INTRODUCTION AND BACKGROUND	2
	2.1 General Introduction	2
	2.2 Background	2
	2.3 The Relationship Between Mutation and Cancer	3
	2.3.1 Heritable mutations	6
	2.4 Mechanism of Carcinogenesis	8
3.	APPROACHES TO THE TESTING OF CHEMICALS	10
4.	CRITERIA FOR SELECTION OF TESTS	15
5.	IN VITRO TESTS	19
	5.1 Bacterial Mutagenicity Tests	19
	5.1.1 Salmonella/microsome assay (Ames assay)	21
	5.1.2 Reverse mutation assay using strains of E. coli WP_2	22
	5.1.3 Tests using E. coli K-12	23
	5.1.4 Forward mutation in other bacterial systems	25
	5.1.5 Tests using Bacillus subtilis	25
	5.1.6 The fluctuation test	26
	5.1.7 The Inductest	28
	5.2 Mutagenicity Tests with Yeasts and Moulds	31
	5.2.1 Tests using yeasts	32
	5.2.2 Tests using Neurospora crassa	36
	5.2.3 Tests using Aspergillus nidulans	37
	5.3 Mammalian Cell Mutagenesis	38
	5.4 DNA Damage and its Subsequent Repair	42

	Page
5.4.1 Unscheduled DNA synthesis (UDS)	44
5.4.2 DNA fragmentation	46
5.5 Chromosome Damage	47
5.5.1 The Sister Chromatid Exchange (SCE) test	49
5.5.2 The Micronucleus test: *in vitro*	51
5.6 Cell Transformation	53
5.6.1 Mass culture	53
5.6.2 Clonal assays	54
5.6.3 Focus assays	55
5.6.4 Soft agar assays	55
5.6.5 Application of transformation assays in screening programmes	56
6. ADVANTAGES AND LIMITATIONS OF THE TESTS FOR SCREENING PURPOSES	59
6.1 Monitoring Industrial Exposure	60
7. SUMMARY AND CONCLUSIONS	63
8. RECOMMENDATIONS	65
9. REFERENCES	66
APPENDIX 1	74
APPENDIX 2: GLOSSARY	85

1. PURPOSE AND OBJECTIVES

Man continues to be exposed to an increasing number of harmful chemicals that find their way into his living and working environments. Therefore, it is essential to identify potential hazards at an early stage, in order to minimize exposure and the associated risk of long-term health effects.

Short-term tests are very valuable in the identification of chemicals that are capable of causing mutation. It has been well-documented that many of these chemicals are also capable of inducing cancer. Thus, the same short-term tests are often used as indicators of the carcinogenic potential of these chemicals.

The objectives of this report are to review the current status of short-term *in vitro* genotoxicity tests with respect to their contribution in evaluating the carcinogenic potential of a given chemical to man and their potential value as a basis for making regulatory decisions.

Information gained from short-term test results can assist in other ways. These include the detection of potential carcinogens in complex mixtures; the development of risk assessment methodology: elucidation of mechanisms of carcinogenesis; the formulation of priority lists of chemicals that should be tested or re-tested in animals; the development of useful non-mutagenic chemicals; the monitoring of human populations for adverse health effects; the provision of additional scientific information to assist in the interpretation of the potential carcinogenicity of a chemical. Such additional information will be touched upon only briefly in this report.

2. INTRODUCTION AND BACKGROUND

2.1 General Introduction

Within the last decade, several study groups throughout the world have generated a large amount of information on the ability of chemicals to cause cancer (Adler, 1980; Dunkel, 1979; Poirier et al., 1979; Zeiger & Drake, 1979). However, the aims of the various test programmes have differed widely. For example, some groups have concentrated on the standardization of laboratory techniques, while others have focused on the provision of test data for specific chemicals or the ability of a given test to detect alterations in normal genetic events. Clearly, the detailed information available on the process of chemical carcinogenesis covers many disciplines and has become an unwieldy subject. Therefore, the information which was extracted from the literature for this report was confined to those studies considered to have direct relevance to the needs of the Alberta Occupational Health and Safety Division. Reduction of the data to a manageable size was achieved by discussion with recognised experts, by including only the most recent publications and by reference to good reviews (which provided more important salient details of earlier studies). This report examines the major issues surrounding the usefulness of given in vitro tests and the currently recognised criteria for selection of short-term genotoxicity tests for the identification of potential carcinogens.

2.2 Background

The closest approach to a unifying theory to explain the actions of carcinogens has resulted from recent developments in genetic toxicology. One of the primary theories for the induction of cancer is based upon the hypothesis that chemical carcinogens interact with the genetic material in somatic cells and alter the cellular behaviour in such a way that cancer develops (Ashby & de Serres, 1981).

The fact that deoxyribonucleic acid (DNA) is the basic hereditary material common to almost all living organisms would theoretically justify the use of any organism in experimental studies. However, those organisms which are considered to be sufficiently similar to man in terms of organisation and complexity are considered to be more suitable for the purpose of evaluating human genetic risk, caused by exposure to various chemicals in the environment. Unfortunately, long-term animal studies do not facilitate the rapid identification and control of environmental mutagens or carcinogens. Research has already shown that many chemical carcinogens are mutagens (Ames, 1979; Hollstein et al., 1979; McCann et al., 1976; Poirier et al., 1979; Purchase et al., 1978; Rinkus et al., 1979). This correlation is the primary reason behind the current widespread investigation of potential carcinogens by the use of various short-term mutagenicity tests. Whilst the development of such tests is fairly recent, it is worth noting that the use of mutation systems to detect chemical carcinogens is more than thirty years old. The recent findings of workers such as Ames complement the conclusions of earlier workers that chemical mutagens might also be mammalian carcinogens. (For historical details see reviews: Mohn, 1981; Mohn & Ellenberger, 1980; Sankaranarayanan, 1981).

2.3 Relationship Between Mutation and Cancer

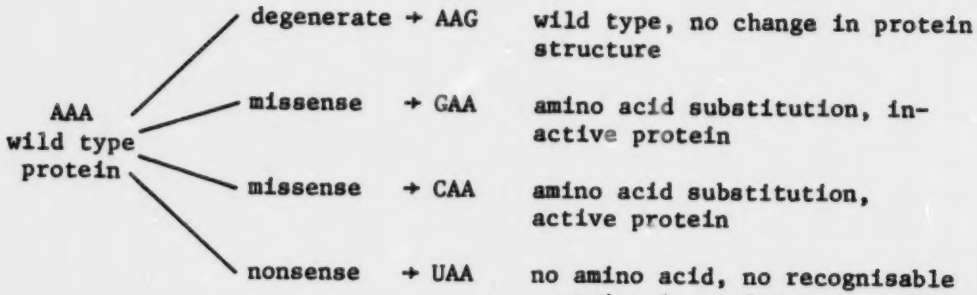
According to Flamm (1978), mutagenicity may be defined as "the ability to damage DNA". It is also important to note that mutational changes in the genetic material can be transferred to the progeny. At the molecular level, chemical mutagens may incite changes in the sequence or number of nucleotide base pairs of the DNA. These alterations are copied in the messenger ribonucleic acid (mRNA) and subsequently are translated as a different amino acid sequence from normal. If amino acid replacements occur in a non-critical portion of the molecule or involve replacement of one amino acid by another of similar size or

property, then the resulting protein may retain "wild type" (normal) activity, despite an alteration in the primary structure. Generally, mutant forms differ from wild type in one or more hereditary characteristics, not all of which may be detected in the phenotype. This results largely from the host's natural "buffering" system against possible deleterious effects of mutation (e.g. degeneracy of the genetic code; see Figure 1).

Base pair substitutions and frameshift (addition/deletion) mutations are termed "gene mutations" or "point mutations", but they are usually induced by distinctly different classes of chemical mutagens. For example, aflatoxin B_1, benzo(a)pyrene and 2-acetylaminofluorine have been detected as frameshift mutagens in bacterial test systems. Mutagens which cause base pair substitutions include β-propiolactone and propane-sultone, and certain chemicals, such as nitroquinoline-N-oxide, cause both types of mutations (McCann & Ames, 1976). Such microlesions are detected as variants of characteristics controlled by specific gene loci or as recessive lethals, unlike macrolesions (e.g. chromosome damage), which can be detected by cytological techniques. Further consideration will be given later to the detection of various end points, when individual tests are discussed.

Clearly, not all mutations will result in the development of cancerous tumours in experimental animals or man. The "mutagenic efficiency" of a chemical is affected by such factors as its concentration, absorption, distribution, stability, penetration in the target cell, reaction with DNA, immunological response and also detoxification and excretion in the host organism (Anderson & Longstaff, 1981). In a similar way, these factors will also influence the carcinogenic potential of a chemical. "A chemical is considered to be a carcinogen, if it leads to a statistically significant increase in the incidence of tumours in experimental animals" (Ashby & de Serres, 1981), but it was stated earlier that such studies do not facilitate the rapid identification and control of environmental mutagens.

Figure 1. Possible phenotypes resulting from one letter mutational alterations of a coding unit (adapted from Hartman & Suskind, 1969)



Therefore, the establishment of a chemical as a mutagen (and by empirical correlation a carcinogen) in reliable short-term screening tests is highly desirable.

Spontaneous or background mutation rates are considered to be sufficient to maintain the genetic variability of the human population (Bloom, 1981). However, induced increases in the mutation rate could lead to a general decline in genetic health. In addition to the identification of potentially carcinogenic chemical mutagens, short-term tests can aid in the identification of chemicals that may cause inherited mutation in man leading to genetic disease. Although there are no mutagens that have yet been shown to cause inherited effects in man, it is wise to assume that such induced effects in animal models can be extrapolated to man (Douglas, personal communication, 1982).

2.3.1 Heritable mutations

The prevention of intergenerational effects of hazardous chemicals is just as important as the prevention of cancer. These effects tend to be expressed phenotypically in the human as a genetic disease.

Chromosomal mutations constitute the most easily defined of the human genetic diseases. A classic example is the widely recognized Down syndrome, a trisomic condition of chromosome 21, which commonly arises from non-disjunction and translocation in either of the parental germinal cells. Chromosomal aberrations may be detected and measured using *in vitro* techniques with somatic cells and *in vivo* techniques with cells from bone marrow or lymphocytes of treated animals. Unfortunately, these end points are neither explicit nor easily interpretable, because assessment is based upon chromosome damage (e.g. breaks and gaps) and cell death and not necessarily of heritable effects. This technique has long been used to identify suspected chromosomal mutagens, but it should be noted that not all clastogenic (chromosome breaking) substances will be capable of producing heritable effects in germinal cells (Flamm, 1978).

Dominant gene mutations are another class of human genetic disease. The dominant mutant trait which is expressed may involve structural anomalies and physical malformations (i.e. disorders), rather than functional alterations. There are some exceptions to this rule among the ∿1,000 dominant mutations known to occur in human populations. The group of known hereditary neoplasms include nine forms of cancer, arising from mutations of dominant genes (single, autosomal genes). Bilateral retinoblastoma is the most well-documented of these cancers, with an incidence of 1:20,000 births (Bloom, 1981). A high mortality rate was associated with all cases, which were therefore recorded as new mutations. Polyposis coli is the most common hereditary neoplasm (1:8,000), and is characterised by numerous polyps of the rectum and colon, which generally develop into carcinomas with increasing age.

The remaining seven hereditary neoplasms are also rare (collective incidence 1:5,000). The incidence of dominant gene mutations (including hereditary neoplasms) is of the same order of magnitude as that for chromosomal mutation and the combination of both is estimated at approximately 1% (Bloom, 1981).

Recessive mutations form the third group to be considered briefly here. Such conditions rely upon the homozygous condition for expression (i.e. the mating of homologous mutant genes). Therefore, an individual may carry a recessive gene mutation without expressing the particular trait (e.g. sickle cell anaemia or phenylketonuria), unless a second mutation occurs which permits the expression of the first recessive mutation.

Many of the mutations that occur in humans are known to be base pair substitutions. It is likely that other molecular mechanisms such as frameshifts and deletions are equally responsible (Flamm, 1978).

The measurement of heritable effects is used as a means of detecting both dominant and recessive gene mutations. It was previously mentioned that bacterial (prokaryotic) test systems will detect point mutations. In addition to the measurement of point mutations, eukaryotic (plant and animal) systems such as

Neurospora will also measure inter and intragenic deletions and gene conversions. The advantages and limitations of each of the test systems will be discussed in detail later in this report. (See Section 5).

Relatively common disorders such as diabetes and epilepsy may also be classified as genetic diseases, because their recurrence in families implies a strong genetic component. However, these conditions are not clearly defined in genetic terms, although it is believed that they are transmitted as multiple gene disorders. It is unlikely that current mutagenicity test methods would have great relevance to this group of disorders (Flamm, 1978).

2.4 Mechanism of carcinogenesis

The development of cancer in humans is a multistage process. Some authors state that three broad phases are currently recognised as the stages of cancer induction (Clayson, 1980), whilst others suggest five or more stages (Brusick, 1980). Irrespective of the number of stages described, only the first two, i.e. initiation and promotion are well known (Farber, 1981; Miller, 1978). Initiation appears to be a mutation or DNA lesion affecting a particular group of genes. The interaction of a carcinogen with DNA is fixed within the genome (through specific metabolic processes or cell replication), such that initiation is an irreversible event (Brusick, 1980; Stoltz in: Stich & San, 1981).

Promotion is thought to involve recombination or gene derepression which facilitate the expression of the initiating event. Certain of these physiological and biochemical processes may be reversible. Cocarcinogens which modify the carcinogenic process can act at any stage, although promoters are known to be effective after the initiating event. The role of cocarcinogens and anticarcinogens in the induction of human cancer is complex, and poorly understood at this time. Therefore,it is important

that research efforts be directed towards the development of short-term screening tests for the detection of co- and anticarcinogens (McCalla, personal communication, 1982; Pitot et al., 1981; Stoltz in: Stich & San, 1981).

The final phase of progression to malignant tumours (neoplasia) is the least understood part of the process. During the latent period, which can range from five to forty years, tumour progression is characterised by loss of homeostatic control (i.e. change of biochemical and immunological properties), resulting in cell proliferation and the development of tumour masses. In the final stage, the dividing cells are transported by the vascular system to various body tissues and malignant tumours may then appear at many sites.

Further development of short-term, in vitro tests and model systems in vivo, which attempt to simulate the stages of cancer induction, should help to define the complex mechanism of carcinogenesis.

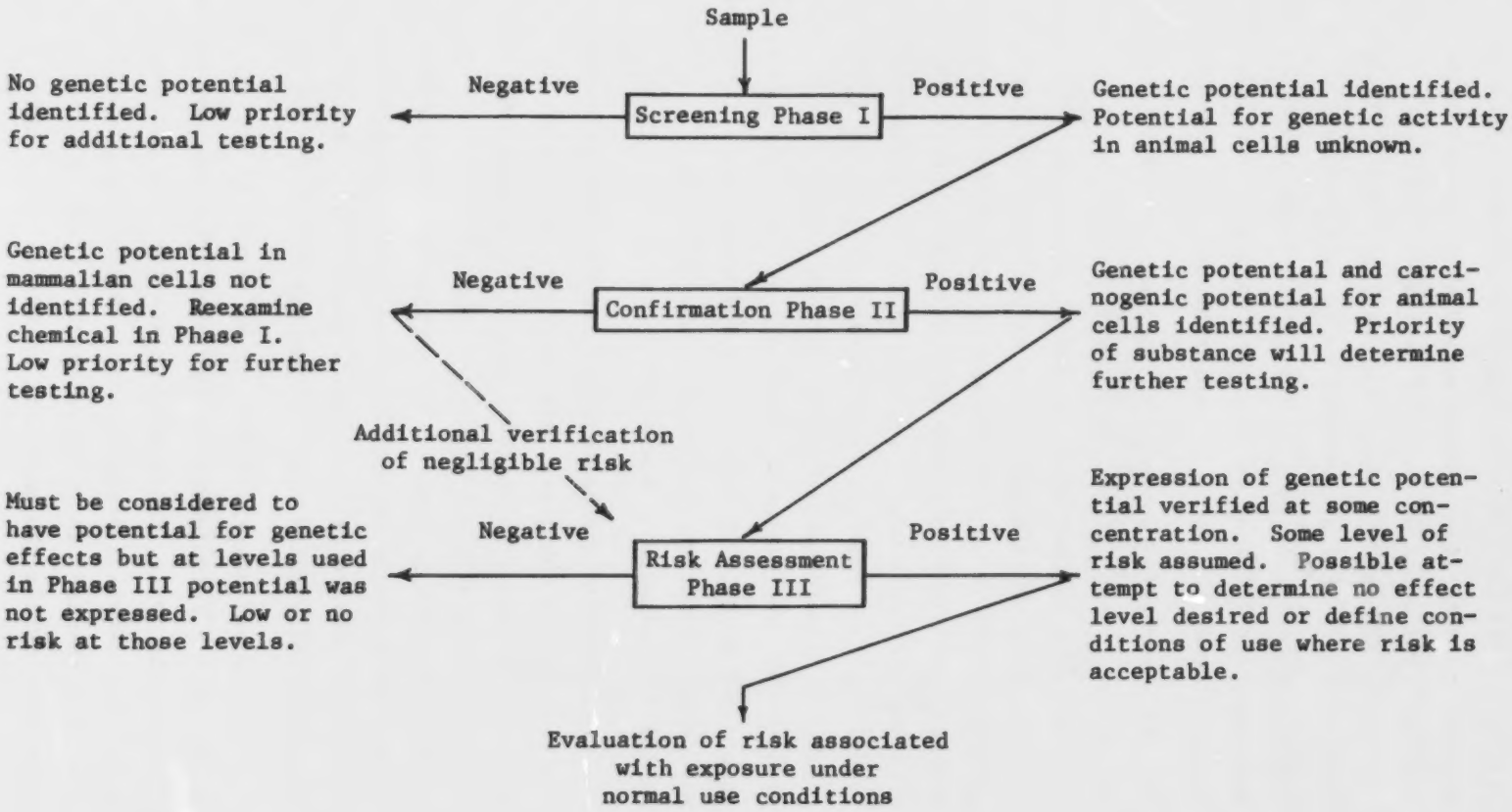
3. APPROACHES TO THE TESTING OF CHEMICALS

The classical approach to carcinogen testing has made use of rats, mice and occasionally guinea pigs, rabbits and hamsters in long-term studies. However, such studies require considerable time, scientific skills (toxicological, veterinary and pathology) and financial resources, if meaningful results are to be obtained. For example, at the present time, tests on a chemical for multiple observational end points may require as much as $300,000 - $500,000 (U.S.) for periods up to 64 months (Weisburger & Williams, 1981). Several short-term tests on the other hand are much less expensive. Brusick (1980) reports that a battery of *in vitro* tests in the U.S. Gene-Tox Programme cost $12,000 - $18,000 (U.S.) per sample evaluated (without confirmation retest) and results may be obtained within ten to twelve weeks.

Methods of testing chemicals for genotoxic properties are both numerous and specific. However, from the literature it is evident that there are two major approaches to the methods of testing, i.e. the tier and battery systems. Tier systems have been adopted most frequently when large numbers of substances require testing at the same time. Ideally, the first tier of tests should be rapid, reliable and inexpensive in order to accomodate all of the substances under test (for details of research opinion see Brusick, 1980; Butterworth, 1979; IARC/WHO Monograph, 1980; Legator in: Stich & San, 1981). Within the limitations of the tests used, the response at this level will eliminate those substances not requiring further evaluation. The smaller number of remaining substances will be subjected to more lengthy, sophisticated tests. This approach is cost-effective, but it is also more time-consuming. One suggested tier system is summarized in Figure 2.

The first phase includes microbial tests for mutation, DNA repair and mitotic crossing-over phenomena. In the U.S. such tests have provided a rapid yet relatively inexpensive evaluation of as many as several thousand substances per year. It is essential that screening tests have uniform protocols, that they are simple and reproducible (for

Figure 2. Tier approach to genetic and carcinogenic testing (from Brusick, 1980)


Sample
Negative
Positive
Screening Phase I
No genetic potential identified. Low priority for additional testing.
Genetic potential identified. Potential for genetic activity in animal cells unknown.
Negative
Positive
Confirmation Phase II
Genetic potential in mammalian cells not identified. Reexamine chemical in Phase I. Low priority for further testing.
Genetic potential and carcinogenic potential for animal cells identified. Priority of substance will determine further testing.
Additional verification of negligible risk
Negative
Positive
Risk Assessment Phase III
Must be considered to have potential for genetic effects but at levels used in Phase III potential was not expressed. Low or no risk at those levels.
Expression of genetic potential verified at some concentration. Some level of risk assumed. Possible attempt to determine no effect level desired or define conditions of use where risk is acceptable.
Evaluation of risk associated with exposure under normal use conditions

trained staff) and that the numbers of false positive and false negative results (i.e. not supported by long-term animal studies) are low. The value of the tier system is critically dependent upon the reliability of the phase I screening assays. Those substances which reach tier II are subject to *in vitro* mammalian cell assays, *Drosophila* tests, or *in vivo* short-term mammalian tests, while those reaching tier III lead to long-term mammalian studies. Data from the latter provide the basis for quantitative risk assessments.

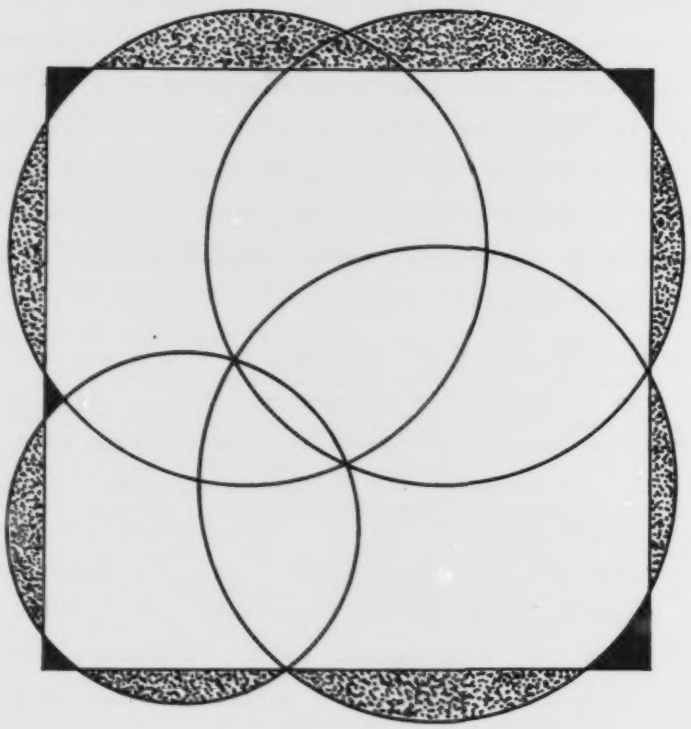
An alternative approach to the tier system is the battery system and at the present time, the battery concept has gained favour over the tier approach to testing programmes. This change of emphasis has been influenced by the timing of data collection, concern about the rate of false positive or negative responses from phase I screening tests, and the need to measure multiple effects (end points). Perhaps a more significant factor, however, was the initially recommended mutagenicity guidelines from the U.S. Environmental Protection Agency (EPA) and other U.S. government agencies. Although a battery of tests provides a simultaneous evaluation of chemicals at different levels, non-genotoxic agents can be quickly eliminated, and potentially hazardous agents that may produce mutation or cancer may be identified in a shorter time period. An example of the battery concept is illustrated in Figure 3.

Each test in the battery has its own limitations when used with a wide range of chemicals, but some overlap will occur for most genotoxic agents. The careful selection of tests for inclusion in a battery system will minimize the number of genotoxic agents that may escape detection because of the technical limitations of certain tests. An adequate battery of tests should contain some redundancy, which will confirm or cross-check results and thereby help to identify the potentially false positive responses.

Biological questions need to be carefully defined before test batteries are formulated, because such batteries may consist wholly of screening tests, tests appropriate for risk assessment or a mixture of both. For example, screening tests are chosen if the carcinogenic potential of a chemical is to be determined (Brusick, 1980). Whilst screening tests will define the genotoxic potential of an agent in prokaryotes,

Figure 3. Battery approach to genetic and carcinogenic testing (from Brusick, 1980)

The square represents the total number of chemicals with mutagenic and/or carcinogenic activity. Each circle represents a specific test and its range of detectability. Overlapping indicates compounds detected in multiple assays. The area of the circles will vary according to the test selected.



Responses designated as false negatives

Responses designated as false positives

they cannot be used as the sole criteria for risk estimation in eukaryotes, especially man, because they exclude information on dose, route of administration, and metabolism. Nevertheless, attempts have been made to use in vitro test results in this way and inevitably they have created considerable confusion.

We know that the structure and function of DNA is very similar in all organisms. Therefore, a test sample which produces a structural or functional alteration in the DNA of a submammalian species theoretically should be capable of producing the same or a very similar response in rodent and human DNA. This assumes that the biologically active form of the test sample reaches the target site and interacts in a similar fashion at this site in the two different species. Under certain circumstances, it is possible that a viable approach to the estimation of genetic risk may combine in vitro mutagenicity data with in vivo chemical data (i.e. pharmacodynamics) showing interactions with germ cells (Brusick, 1980).

The development of carcinogenicity risk assessment models using results from a combination of submammalian, in vitro mammalian and in vivo somatic tests is more justified than relying upon any single in vitro test. For example, the induction of point mutations in mammals, especially mammalian germ cells, cannot be assessed in practical terms by current methods in genetic toxicology. The measurement of chromosome alterations is the basis of the most practical of the in vivo models (Soares in: Butterworth, 1979). Therefore, the potential for induction of point mutations of a particular test sample may be better defined by a careful selection of the appropriate submammalian (Drosophila) and in vitro mammalian assays.

4. CRITERIA FOR SELECTION OF TESTS

The major criteria to be considered in the preparation of battery systems are tests with reliable end points, accuracy, and clear biological significance, that is relevance to mutagenicity and carcinogenicity (Anderson in: Berg, 1979; Brusick, 1980; IARC/WHO, 1980). An objective end point will enhance the reproducibility of a given test (Kier in: Banbury Report No. 2, 1979). In addition, the metabolic reactions underlying all tests should be optimized, in order to obtain maximum relevance to intact mammalian systems. In some cases, intact cells may be preferred over S9 rodent liver microsome homogenates for metabolic activation (Weisburger & Williams, 1981). For example, the use of hepatocytes is believed to reflect more closely the in vivo conditions, because hepatocytes retain the full complement of drug metabolizing enzymes without the addition of cofactors. Damage to drug metabolizing enzymes (i.e. cytochrome P450 system) may occur in the preparation of S9 liver homogenates and therefore the addition of cofactors becomes necessary, in order to optimize oxidation and conjugation activity (Hubbard et al. in: Ashby & de Serres, 1981). Thus, the usefulness of a given test can be enhanced by the presence of an intact metabolic system with its more reliable end points (Williams in: Stich & San, 1981). Tests which incorporate a metabolic system not only extend the range of identification of mutagens and non-mutagens, but may also provide a measure of the relative mutagenic potency of chemicals, i.e. ranking of chemicals as strong or weak mutagens in short-term tests (Mohn & Ellenberger, 1980). However, this does not provide a measure of mutagenic potency in man.

Tests which can detect more than one end point reliably are even more desirable for use in battery systems, because they can be used to verify results from other tests and also minimize redundancy of a given test system. In this way, false positive results can be cross-checked without the addition of further tests which would significantly increase costs. It is important to include tests that will together measure a broad spectrum of mutational events and yet respond accurately to a wide variety of chemical classes (See Figure 4).

Figure 4. Examples of some environmental agents likely to be missed using *in vitro* assays without redundancy (from Brusick, D., 1980)

Compound	Missed in	Detected in	Type of end point induced
Hexamethylphosphoramide	Ames	Mouse lymphoma	Gene mutation
Natulan	Ames	Mouse lymphoma	Gene mutation
Acrylonitrile	Ames*	*E. coli* WP_2	Gene mutation
Diethylstilbesterol	Ames	Mouse lymphoma	Gene mutation
Urethane	Ames and Mouse lymphoma	*Drosophila* SLRL	Gene mutation
Formaldehyde	Ames*	Mouse lymphoma	Gene mutation

*Some protocol modifications have been reported to increase the utility of this assay for specific compounds.

Several classes of chemicals which are known to be mutagenic from animal studies (e.g. diethylstilboestrol) remain undetected or produce a weak response in some of the standard short-term, in vitro tests. Structure activity analysis of specific chemicals within a class under investigation can be of value in ensuring that testing is cost-effective; for example, dimethoxybenzidine may be suspected as a potential carcinogen because it is structurally similar to the known carcinogen benzidine (Ashby, 1980). Therefore, systems should be calibrated carefully using known carcinogens and non-carcinogens as positive and negative controls (Hollstein et al., 1979).

Well-defined test protocols are necessary, because standardization helps to ensure that reliable, reproducible results are obtained and this in turn assists in the choice of tests for regulatory purposes. The predictive value of individual tests has been defined as 'the proportion of true carcinogens among all compounds giving positive results.' However, this criterion for comparison of assay performance has limited usefulness because it does not describe the reliability of a given test (Cooper II et al., 1979; Kretzer et al., 1979). More often, test methods are compared by criteria such as sensitivity (the true positive or carcinogen fraction), specificity (the true negative or non-carcinogen fraction) and accuracy (the number of correct positive and negative responses in the total number of agents tested) (Ashby & de Serres, 1981; Kretzer et al., 1979; Purchase et al., 1978).

Other desirable features in a test system include quantitative sensitivity, which will be very significant in the detection of active impurities in chemicals or mixtures (e.g. active substances at low concentrations in the environment). In the long term, a highly desirable feature of an in vitro test would be the ability to provide dose-response data that can be used reliably and meaningfully for extrapolation to man. At present, however, data from in vivo systems are thought to be more reliable and therefore continue to be used as the basis for risk-benefit estimates (IARC/WHO, 1980; Wolff, 1977).

Many of the short-term tests are not well validated and therefore their advantages and limitations are not fully appreciated at

this time. Current research may produce more useful tests and therefore a battery system which is flexible will readily incorporate improved testing methods. In summary, practical assay systems are required to be sensitive, relevant and both time and cost efficient.

5. IN VITRO TESTS

The majority of tier and battery systems utilize tests chosen from seven groups of tests, i.e. bacterial mutagenesis, eukaryote mutagenesis, Drosophila mutagenesis, mammalian cell mutagenesis, DNA damage, chromosome damage and oncogenic (malignant) transformation. The most relevant in vitro screening tests will be considered here.

5.1 Bacterial Mutagenicity Tests

Bacterial mutagenicity tests have been widely used in test batteries because results are rapidly obtained, easily reproduced and the techniques are relatively simple for experienced workers.

For primary identification purposes the most commonly used bacterial species are Salmonella typhimurium and Escherichia coli. The genetics of these bacteria are well understood and further genetic manipulation of mutant strains has improved their sensitivity over that of the wild type to a wider range of chemical mutagens.

Recall, that each mutagenic agent produces a characteristic mutational spectrum, depending upon the nature of the primary DNA alteration (e.g. base pair substitution or frameshift) and the subsequent effects of DNA repair and replication. Therefore, a mutagen may create different mutational spectra in different bacterial species and strains.

Practical tests may detect both forward and reverse mutations. In theory, forward mutations (e.g. from antibiotic or heat sensitivity to antibiotic or heat resistance) present a large genetic target and should permit the detection of a wide range of mutagenic effects (e.g. base pair substitutions, frameshifts, additions, deletions). It should be noted that the control of resistance may be restricted to a few genes and in this case such a forward mutation has a limited genetic target.

Reverse mutation assays use mutant bacterial strains and the rate of back mutation to prototrophy (ability to grow on minimal medium, i.e. wild type) is scored in the test. Reversion testing is very specific (cf. forward mutation). A true reverse mutation occurs at the same locus as the forward mutation and hence the genetic target for the mutagen is very small. Occasionally, a second mutation may occur (at a different locus) that masks the effect of the original mutant genotype. Such a "suppressor mutation" may be separated out by back-crossing the revertants to wild type. Reverse mutation tests detect base pair substitutions and frameshifts but not additions or deletions of more than one nucleotide base.

Unless the biotransformation of chemical compounds is taken into account in the in vitro tests, many chemical mutagens will go undetected. The effects on target organs are controlled by the pharmacodynamics of a given chemical (i.e. its metabolism, half life and breakdown within the mammalian system). In order to simulate mammalian metabolism in bacterial tests, microsomal enzyme preparations from mammalian organs such as the liver have been added to the test system. The use of activating enzymes from tissues other than liver offers no significant advantage because results with liver generally reflect activity detected in other organs (e.g. lung or kidney). The variation in activation appears to be more quantitative than qualitative. The overall efficiency of bacterial test systems has been improved in this way, but the tests do not provide quantitative assessment of mutagenic potency in man. For example, they do not account for the differences between the in vivo metabolic pathways.

Alternatively, the host-mediated assay takes into account mammalian metabolism in vivo and may be considered more comparable with normal mammalian metabolism of chemicals. Briefly, the bacterial indicator strain is injected into the peritoneal cavity, the circulatory system or the testicles of a given animal. The host animal is then dosed with the test substance

and the indicator strain (usually S. typhimurium or E. coli) is recovered from the host and examined for mutation using an in vitro assay (bacterial plate technique). Whilst a positive result may indicate the likelihood of a positive mutagenic reaction in the whole animal, this is still an indirect method of mutation induction and the influence of mutagens on DNA repair mechanisms of the mammal will not be detected (Sankaranarayanan, 1981).

5.1.1 Salmonella/microsome assay (Ames assay)

This test was developed by Dr. Bruce Ames in 1973 as a screening test. The standard Ames assay employs a combination of differently induced histidine auxotrophic (nutritionally deficient) mutants of S. typhimurium. The test detects revertants to histidine independence, i.e. wild type. Several indicator strains have now been developed to overcome mutagen specificity (e.g. specific strains for base pair substitution are TA1535 and TA100; frameshifts - strains TA98, TA1537, TA1538). All of the test strains now used have a cell wall defect, i.e. the strains contain an additional mutation, which causes partial loss of the lipopolysaccharide layer, thereby permitting diffusion of large organic molecules such as polycyclic hydrocarbons, aflatoxins and many aromatic amines into the bacterial cell. Further sensitivity is conferred upon tester strains by a mutation which causes loss of the excision repair system, so that certain primary DNA lesions remain unhealed. Some strains (TA100, TA98) contain a plasmid, pKM101, that increases error-prone repair (i.e., produces mutation because of faulty repair synthesis) and also enhances the sensitivity of the strains to reversion by carcinogens such as benzo(a) pyrene, aflatoxin B_1, and various nitrofurans. Sensitivity in this context describes the increased susceptibility of tester strains to DNA damage. It should be noted that bacterial systems which are devoid of normal component parts do not reflect mammalian cells nor in vivo mammalian conditions. Nevertheless, they are adequate for use in screening tests.

Several known classes of mutagens are not detected in bacterial systems (e.g. nitrosamines, 2(β)-naphthylamine, cyclophosphamide), because the parent compound does not elicit the mutagenic event. Many carcinogens must be metabolised to more reactive electrophilic metabolites before DNA damage occurs (Miller, 1978). Thus, metabolic activation systems are required in bacterial tests and hence this explains the use of mammalian liver homogenates (rodent S-9 fraction) in the Ames assay. As the cytochrome P450 system has been extensively studied and the liver is the first major organ to encounter a carcinogen, then it is the most logical organ to choose for the preparation of microsomal enzymes for test purposes. Known direct-acting mutagens such as alkylating agents and acylating agents may be readily detected in the Salmonella/microsome test.

There is general agreement that the S. typhimurium series is capable of detecting most environmental mutagens and carcinogens (Ames, 1979 (see Appendix I); Hollstein et al., 1979; McCann et al., 1976; Nagao et al., 1978).

5.1.2 Reverse mutation assay using strains of E. coli WP_2

The E. coli tryptophan reversion test detects true reversions (base pair substitution) at the mutant site and also suppressor mutations at a different locus. A series of strains are used that have altered DNA repair capacity.

Ames et al. (1975) recommended the use of E. coli WP_2 and E. coli WP_2 uvrA strains as complementary strains for the Salmonella/microsome assay, because at that time some chemicals such as nitrofurans were active only in the E. coli test. Since then, the sensitivity of S. typhimurium strains (TA1535 and TA1538) has been improved by addition of plasmid pKM101 (new strains TA98, TA100). Plasmid pKM101 has also been added to an excision repair strain of WP_2, which will now reliably detect a variety of mutagens (e.g. alkylating agents, polycyclic aromatic hydrocarbons, azo dyes). Physical carcinogens such as asbestos are equally inactive in both the E. coli WP_2 test and the Ames assay, perhaps because

the size of the fibres renders any meaningful testing with bacteria impossible (Mohn, 1981). Reverse mutation systems have produced comparable results by using carefully selected strains of either E. coli or S. typhimurium (Ashby & de Serres, 1981).

It has been suggested that WP_2 tester strains are best used in an integrated bacterial mutation test in combination with several of the S. typhimurium strains (Davis, Devoret et al., Report 2 in IARC/WHO, 1980). However, in a detailed evaluation of the E. coli WP_2 assay (Brusick, Simmon et al., 1980) the general conclusion of the working group was that the E. coli WP_2 reverse mutation system was a valuable tool for mutagenesis research, but that there was no evidence from the literature that this assay would contribute significantly to results obtained from careful application of the Ames Salmonella/microsome assay.

5.1.3 Tests using E. coli K-12

Chemical mutagens may be screened using a number of mutation systems in strains of E. coli K-12 which include specific back mutation systems from auxotrophy to prototrophy, such as strain ND160 which is sensitive to mutagens such as caffeine and steroidal diamines. Equally useful are systems which detect different classes of base pair substitutions (strain AB1157) and the tryptophan auxotrophic mutants that can detect base pair changes and frameshifts. In addition to reversion testing, a number of forward mutation tests have been developed with wild type strains (e.g. resistance to antimetabolites such as phenytyl alcohol, nalidixic acid) and mutant strains in which further mutations are detected (e.g. resistance against sugars such as galactose). The induction of suppressor mutations to streptomycin independence in Sd-4 strains has also been used to screen many chemicals and this has provided some quantitative information on the relationship between mutagenic potency and chemical reactivity of various agents.

The molecular changes induced by chemicals in a forward mutation assay can now be detected, as a result of the recent

determination of DNA base sequence in the lac i gene, which correspond colinearly with amino acid sequences in the lac repressor protein (Davis, Devoret et al., Report 2 IARC/WHO, 1980).

A strain (343/113) has been developed that will simultaneously detect forward mutations and several specific back mutations and is sensitive to a large variety of mutagens. Currently, genetic effects can be scored as forward mutations in the gal R gene and in several genes that lead to resistance to 5-methyltryptophan. Reversion testing includes $arg^-_{56} \rightarrow arg^+$ (base pair substitution), $nad^-_{113} \rightarrow nad^+$ (acridine induced frameshifts) and $leu^-_{18} \rightarrow leu^+$ (polycyclic hydrocarbon induced frameshifts). DNA repair-deficient strains and plasmid pKM101-containing strains are also used to improve the sensitivity of the test system.

Liquid suspension tests using E. coli 343/113 are used to confirm plate assay results and to quantify mutagenic potency in different bacterial strains, rather than to prescreen for mutagens.

Another screening test is based upon a differential killing assay in which a strain deficient in DNA repair and its repair-proficient counterpart are treated with a test compound. If the repair-deficient strain is killed to a greater extent, then this is evidence that the compound may have DNA damaging activity. One of the best known of such assays is the pol test in which wild type E. coli K-12 and a strain containing a DNA polymerase I deficient mutant has been used extensively to detect a large number of carcinogens. One disadvantage is that repair-deficient mutants show weaker growth than wild type strains. The pol test was originally developed as a spot test but a broth culture technique has proved a more reliable technique than spot testing with inoculated paper discs or wells cut into agar plates. These tests are generally non-specific and in theory they should detect any lethal or mutagenic interaction of chemicals with DNA that can be repaired by the DNA repair systems (for further discussion of other repair-deficient strains of E. coli see Hollstein et al., 1979). See also Leifer et al. (1981), for a detailed evaluation of tests using DNA repair-deficient bacteria for predicting genotoxicity and carcinogenicity.

5.1.4 Forward mutation in other bacterial systems

A few forward mutation assays have been used for screening environmental chemicals. These include a test using a strain of S. typhimurium to detect a forward mutation to resistance to azetidine carboxylic acid. However, this test offers no advantage over the Ames reversion test. A strain of S. typhimurium derived from a spontaneous his^+ revertant of strain TA1535 can be used to detect a forward mutation to 8-azaguanine resistance. A plasmid (pKM101)-containing derivative of this strain was used in a forward mutation test which compared favourably with the Ames Salmonella/microsome test in the detection of sixteen mutagens. Another strain of S. typhimurium, SV_3, has been used to detect a forward mutation to arabinose resistance and positive results have been recorded for a number of mutagens such as mitomycin C (Anderson & Longstaff, 1981; Davis, Devoret et al., in Report 2, IARC/WHO, 1980; Hollstein et al., 1979).

5.1.5 Tests using Bacillus subtilis

The genetic markers of B. subtilis are as well suited to combined genetical and biochemical studies as those of S. typhimurium and E. coli. Wild type B. subtilis can synthesise all its own amino acids and vitamins and is easily grown on unsupplemented minimal medium. Auxotrophic mutants requiring different amino acids for growth are readily obtained. Therefore, the B. subtilis system offers a range and flexibility equivalent to that of S. typhimurium or E. coli in screening tests and also provides a useful comparison from a different phylogenetic species.

Strains of B. subtilis have been used in plate assays to detect forward mutations at specific loci and DNA repair-deficient strains have been used to score preferential growth inhibition.

Thirty carcinogens were screened using the methionine and histidine auxotrophic strain of B. subtilis TKJ511, which is also an excision repair-deficient mutant. Thirteen of the seventeen known carcinogens were positive and all of the non-carcinogens were negative (Hollstein et al., 1979).

The rec assay using a recombination-deficient strain of B. subtilis M45 is based upon the same principle as the pol test in E. coli K-12. Recent improvements to this spot test involve the use of spores instead of vegetative cells and the addition of an external metabolic activation system (S9 fraction). The chemicals tested in this system include ethyl methane sulphonate (EMS), mitomycin C and hydroxylamine, numerous pesticides, mycotoxins, chemical dyes, and metals. Results were variable and in some tests few positive responses were obtained, for example only twelve of one hundred and fifty pesticides were positive (for details see Hollstein et al., 1979).

B. subtilis is an aerobic, spore-forming bacterium, which is relatively innocuous and easily cultivated. The induction of mutant non-sporing colonies of B. subtilis forms the basis of another less frequently used screening test. Scoring of mutant colonies (lacking brown pigment) may be hampered by the density of wild type colonies on the plate. This factor renders the test less suitable for routine purposes, but it does provide additional information. For example, positive results have been obtained for mitomycin C, 8-methoxypsoralen and also aflatoxin B_1 in the presence of S-9 fraction (Hollstein et al., 1979).

5.1.6 The fluctuation test

The fluctuation test offers the advantage of detecting mutagenicity in microorganisms, using much lower doses of test chemical than the standard plate test. Auxotrophic strains of bacteria, yeasts or fungi are exposed to the test chemical (with or without the addition of a metabolic activation system) and then the exposed cultures are diluted to a lower concentration and dispensed into a series of tubes (10 to 100) containing a nutrient broth. Continued incubation (up to 96 hours) permits growth to saturation and positive tubes are recorded (turbidity).

Generally, this test has been applied to the screening of direct acting mutagens, although a few promutagens have been tested (e.g. benzo(a)pyrene; 7,12-dimethylbenzanthracene). It

has also been of value in detecting low levels of certain direct acting mutagens, which produce a weak or negative response in the standard plate assays. For example, a positive result was obtained for dichlorvos in the fluctuation test, using a tryptophan auxotroph of *E. coli* WP_2, but at a concentration of less than 1/100th of that required for positive results in the standard plate assay. Other chemicals which were positive in this test include methyl methane sulphonate (MMS), dipotassium chromate and mitomycin C. A strain of *S. typhimurium* (TA1535) that produced a negative response with three nitrofurans in the plate assay produced positive results using the fluctuation test. In addition, a positive mutagenic response was obtained for strain TA100 at a much lower concentration than that required for a positive result in the plate test. Another advantage of the fluctuation test is that a mutagenic concentration of the test chemical may be selected that is not further diluted as in the pour plate method (Hollstein et al., 1979).

More recently, the fluctuation test was used to detect the mutagenic activity of forty-two coded chemicals in an international collaborative study (Ashby & de Serres, 1981). *S. typhimurium* strains TA98 and TA100 were used under three different test conditions, i.e. 1) without a metabolic activation system; 2) with S9 fraction; and 3) with fresh rodent (rat) hepatocytes (from uninduced animals). Fifteen of twenty-five known carcinogens tested were positive and ten of the known non-carcinogens were negative. Certain false negatives resulted from inadequate metabolic activation on first testing and the others were presumed to be carcinogenic via a non-mutagenic mechanism. A few anomalous results could not be explained.

A "Microtitre" fluctuation test has also been developed and the qualitative results from this test compare favourably with results from the standard fluctuation test. Quantitative comparisons were not possible because of differences in test protocols (e.g. source of S9), but such disadvantages are common to many microbial test systems (Gatehouse, 1981).

The fluctuation test is more elaborate than the Ames test and it may therefore be considered less attractive for routine purposes. It does, however, offer more flexibility with respect to activation under different conditions and at the present time it provides a useful cross-check on false negative results arising from inadequate metabolic activation in other tests (e.g. plate assays).

5.1.7 The Inductest

Many bacteria are lysogenic, that is their genome carries the DNA of a virus (bacteriophage) in a latent or prophage state. The lambda (λ) phage of E. coli K12 has been widely studied.

The induction of prophage λ is related to a mutagenic action that induces the 'SOS' DNA repair system. SOS is a response to DNA damage that cannot be repaired by the normal excision or post-replication repair processes and it appears to be confined to prokaryotes. Error-prone repair in surviving cells is often recorded. Chemicals that induce prophage may also be carcinogenic (Thomson in: Ashby & de Serres, 1981).

The mutagenic activity of a number of chemicals has been determined by induction of the prophage, i.e. the stimulation of the lytic cycle in bacterial cells carrying latent (dormant) bacteriophages, resulting in the destruction or deactivation of the phage "repressor". The repressor protein is the product of the prophage CI gene and it prevents transcription of the prophage genes by binding to the two operator sites on either side of the CI gene, thus maintaining the latent condition of prophage λ.

Lysis of the host bacterial cells quickly releases many phage particles which are detected when the induced cells are mixed with indicator bacteria and plated out onto nutrient agar. The viruses released from the induced lysogenic cells lyse the indicator bacteria and form plaques (clear zones) on the lawn (confluent growth) of indicator bacteria. The ratio of the number of plaques formed to lysogenic cells present before exposure is a measure of lysogenic induction.

Tester strains of lysogenic E. coli K12 have been improved by the introduction of a mutation (envA) that increases permeability to many mutagens and uvrB mutation that inactivates the excision repair mechanism. Improved procedures include the addition of a mammalian metabolic activation system and it is possible to test for lysogenic induction and mutagenesis within the same cell. Both the mutagenic and inducing activities of aflatoxin B_1, EMS and MMS have been reported using this combined protocol (Davis, Devoret et al., Report 2, IARC/WHO, 1980; Hollstein et al., 1979).

The inductest has been used to screen a number of chemicals (with and without S9). In a screening study of twenty chemicals (including eighteen known carcinogens), only three were positive, i.e., MMS, benzo(a) pyrene and N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG). This demonstrates that the test is not sufficiently reliable for routine use.

A more recent biochemical test for prophage induction that measures induction of enzyme activity (e.g. galactokinase or β-galactosidase) has been developed. The gene for the enzyme is limited to the λ DNA such that the λ repressor blocks enzyme synthesis. When the repressor is deactivated, the enzyme is synthesised and enzyme activity indicates DNA damage. This test has the advantage that results may be obtained in less than 24 hours.

The inductest cannot be considered as a satisfactory alternative to the Ames Salmonella/microsome assay, but it has provided additional positive reactions (e.g. O-toluidine, auramine, mitomycin) that the Ames test has not detected. In addition to rapid results and the use of fewer tester strains, another advantage of the inductest over mutagenicity tests is that a positive response can be observed in the majority of a cell population and toxic effects do not interfere with test results (i.e., positive results do not depend upon bacterial survival of test chemical exposure) (Davis, Devoret et al., Report 2, IARC/WHO, 1980; Thomson in: Ashby & de Serres, 1981).

Some examples of the various bacterial mutagenicity tests that have been discussed in this section are shown in Figure 5.

Figure 5. Examples of some bacterial mutagenicity tests

Test	Bacterial species	End point
Reverse mutation	Salmonella typhimurium TA tester strains (his^-)	His^+ colonies
	Escherichia coli WP2 ($tryp^-$)	$Tryp^+$ colonies
Forward mutation	Salmonella typhimurium (TA 1535 derivative)	8 - Azaguanine resistance
	Bacillus subtilis	Brown colonies
Differential killing	Escherichia coli K-12 pol A^- Bacillus subtilis M45 rec^-	Zone of inhibition
Fluctuation test	Salmonella typhimurium (his^-) (TA 1535)	Growth (turbidity) of mutants
	Escherichia coli WP2 ($tryp^-$)	

5.2 Mutagenicity Tests with Yeasts and Moulds

There are several advantages of using certain species of yeasts and fungi in short-term screening tests to detect carcinogenic compounds. In addition to ease of cultivation and well understood genetic systems, they are eukaryotic and they provide the simplest and most rapid methods for detecting several kinds of mutagenic chromosome effects (not detected in prokaryotes).

The species most commonly used for the detection of point mutations are the yeasts Saccharomyces cerevisiae, Schizosaccharomyces pombe and the Ascomycete fungi Neurospora crassa and Aspergillus nidulans. Numerous strains and genotypes of each of these organisms are commonly used for assaying chemical carcinogens (see Loprieno et al., 1980 for tabulated data).

A wide range of genetic end points may be detected using these organisms. Such end points include dominant lethality, forward mutations for base changes or deletions, reverse mutations for base substitutions or frameshifts, gene conversion, inter- and intragenic recombination, chromosome non-disjunction, translocation, other chromosomal aberrations and mutations in mitochondrial DNA. Certain of these end points (i.e. dominant lethality, translocation and partial or total loss of mitochondrial DNA) are not so readily detected, because their identification is a two step process and therefore they are not entirely suitable for simple, rapid, mutagen testing (Loprieno et al., 1980; von Borstel, 1981).

As the fungi have some P450 mixed-function oxidases, the addition of an exogenous metabolic activation system is not always required. Unfortunately, the fungal tests do not reliably detect procarcinogens, with or without exogenous metabolic activation and very often a high concentration of test chemical is required for induction of mutations (c.f. bacteria). Limited permeability of target cells may be an important factor here (although some authors disagree) and compared with bacteria the

yeasts and moulds show greater metabolic detoxification and they have more DNA repair pathways (Hollstein et al., 1979; Loprieno et al., 1980).

5.2.1 Tests using yeasts

Both haploid and diploid yeast strains have been developed for detecting environmental mutagens and each strain has certain advantages. Haploid strains are used to test for mutation and killing only, and the range of mutagens detected differs from that identified by diploid strains.

The same basic principles of forward and reverse mutation assays described for the bacteria (see section 5.1) are applicable to assays using various strains of Saccharomyces cerevisiae and Schizosaccharomyces pombe. For example, mutations at each of the two genetic loci (ade 1; ade 2) that control adenine biosynthesis have been induced in strains of both species. The mutant colonies are identified by change of pigment from white to red. Methionine auxotrophs of S. cerevisiae have been used in reversion assays to detect base pair substitutions or frameshifts. A forward mutation system to canavine resistance has also been developed in S. cerevisiae (strain XΛ4-8C). Certain direct acting mutagens such as methyl methane sulphonate (MMS), nitrogen mustard and also hydrazine have been detected in this system. In the presence of S9 fraction, other agents were also mutagenic, i.e. N-nitrosopiperidine and several methylated derivatives. Work on the modification of culture conditions to enhance endogenous levels of P450 enzymes has also been undertaken, in order to further improve this test (Hollstein et al., 1979). See Figure 6 for some examples of haploid strains and genotypes of S. cerevisiae commonly used for assaying chemical carcinogens.

Diploid strains of S. cerevisiae, such as D3 are used to detect chemically induced mitotic crossing-over. Again, colony pigmentation facilitates scoring and recombinants that are homozygous at the ade 2 locus form red colonies. The induction of

Figure 6. Examples of haploid strains and genotypes of S. cerevisiae commonly used for assaying chemical carcinogens. (Adapted from von Borstel, 1981)

Strain	Genotype	Genetic Endpoint
XV185-14C	a trp5-48 arg4-17 lys1-1 ade2-1 his1-7 hom3-10	Reversion of ochre, missense and putative frameshift
XV1000-1A	a ade2-912 arg4-17 his1-7 hom3-10 trp5-48	Forward mutation, reversion of ochre, missense and frameshift
6126/16C	a his4-1 ade1-10 arg4-27 tyr7-1 trp1-1	Reversion of ochre, amber, missense and putative frameshift

mitotic crossing-over in strain D3 (with and without S9 mix) was examined using more than one hundred known carcinogens and non-carcinogens. All of the twenty-one known carcinogens were positive, although only seventeen of forty-eight procarcinogens were detected and most aromatic amines, except N-acetoxy-2-AAF were negative. Other variable results were obtained (not supported by long-term animal studies). In a similar exercise, aflatoxin B_1 was positive, but several other mycotoxins were negative. However, a few pesticides and metallic compounds, which were not detected in bacterial mutagenicity assays, were recorded as recombinogenic in S. cerevisiae D3 (Hollstein et al., 1979). Despite the fact that strain D3 is less sensitive to environmental mutagens, more chemicals have been tested with this strain than with any other strain of yeast (von Borstel, 1981).

Gene conversion in strain D4 is scored by loss of either adenine or tryptophan growth requirements. This system has not been extensively validated, although a number of carcinogens have been tested. To date, all mutagens tested have induced mitotic gene conversion (Loprieno et al., 1980).

Strain D5 has a high endogenous cytochrome P-450 activity and therefore exogenous metabolic activation is not always required. D5 is a stable yeast strain that can detect a range of end points. These factors enhance its value for inclusion as a test system in batteries of short-term tests.

A less stable, but more sensitive strain used for testing environmental mutagens is D6. This strain was developed primarily to use non-disjunction as an end point, but mitotic intergenic recombination, gene conversion and mutation may also be detected.

A number of other diploid strains are available for use in short-term screening tests (Loprieno et al., 1980; von Borstel, 1981). Some examples of those discussed here are shown in Figure 7.

Figure 7. Examples of diploid strains and genotypes of S. cerevisiae commonly used for assaying chemical carcinogens. (Adapted from von Borstel, 1981)

Strain	Genotype	Genetic Endpoint
D3	CYH4 ade2 his8 + + +	Mitotic recombination
D4	a gal2 ade2-2 trp5-12 a + ade2-1 trp5-27 leu1 +	Intragenic recombination
D5	a trp1 ade2-40 MAL1 + a + ade2-119 + MAL4	Mitotic recombination, gene conversion and mutation
D6	a his4 tifade3 leu1 trp5 cyh2 met13 ade2-40 a + + + + + + ade2-40	Mitotic non-disjunction
D7	a ade2-40 trp5-12 ilv1-92 1 ade2-119 trp5-27 ilv1-92	Intragenic recombination, induced mitotic crossing-over, gene conversion and reverse mutation

The efficiency of yeast assays in detecting potential carcinogens can be improved by applying test chemicals to cells in both the stationary phase and the vegetative phase. Although a higher concentration of chemical is usually required for an individual test than would be used in the Salmonella/microsome test, some authors believe that the yeast assay is more efficient for carcinogen detection. To date, positive results have been obtained for every human carcinogen tested in yeast assays (von Borstel, 1981).

5.2.2 Tests using Neurospora crassa

N. crassa may be used in both forward and reverse mutation assays. The induction of point mutations at the ad-3 locus includes genetic changes resulting in the production of recessive lethal mutations. Forward mutations in the ad-3A and ad-3B genes of the ad-3 locus, which codes for adenine biosynthesis, result in a requirement for adenine, which is morphologically characterised by the concentration of a purple pigment in the mycelial vacuoles (i.e. mutant colonies are purple). As the loci are so closely linked, further tests (complementation) are required to determine the nature of the alterations (i.e. base pair changes or frameshifts).

Several carcinogens have been detected in this system (e.g. MMS, aflatoxin B_1) and the addition of an exogenous metabolic activation system has extended the capability of the test. However, the system has not been tested with a large number of chemicals.

Strains N23 and N24 are used in reversion assays. Test methods applied to bacterial mutagenicity tests (spot, plate and suspension techniques) can be applied to these strains of N. crassa. Several chemicals have been tested in this system (e.g. alkylating agents, benzo(a)pyrene, nitrofurans). Results of testing a series of chemicals in both the forward and reverse mutation assays compared favourably. Again, the test was improved by the addition of S9 mix (Hollstein et al., 1979).

It appears that these systems have been widely used for testing chemicals (Brown et al., 1979). However, the performance of these tests requires a higher level of expertise in comparison with the bacterial mutagenicity assays, which renders them less attractive for routine purposes.

5.2.3 Tests using Aspergillus nidulans

Forward and reverse mutations and small deletions in groups of genes may be detected using haploid strains of A. nidulans, but diploid strains have also been constructed for the detection of mitotic crossing-over and mitotic non-disjunction.

The induction of mutants resistant to chemical compounds that inhibit the growth of wild type colonies are forward mutations, (e.g. to 8-azaguanine resistance or para-fluorophenylalanine resistance).

In the diploid heterozygous strains P and P1 growth will occur on medium containing p-fluorophenylalanine (and is scored using colony colour markers), if mitotic crossing-over or non-disjunction has occurred in a specific part of the chromosome. Some useful results have been obtained from this test, e.g. pesticides such as dichlorvos and benomyl were positive. In another test for non-disjunction, a positive response was recorded for griseofulvin (usually negative in most short-term mutagenicity tests).

At this time the haploid Aspergillus test systems have a wider data base than the diploid tests. Further validation of both series of test systems and improved standardization (e.g. metabolic activation) will enhance their value for routine purposes. The information provided, tends to complement that derived from bacterial mutagenicity tests (Käfer et al., 1982; Scott et al., 1982).

5.3 Mammalian Cell Mutagenesis

Mutagenesis assays using mammalian cells complement microbial assays, because they detect some known carcinogens that have not been readily identified in the microbial tests, (eg. acetemide, 1,2-dimethylhydrazine). These mammalian tests appear to be useful additions to test batteries, although currently the individual assays have not been extensively validated.

Established cell lines with stable karyotypes (chromosome constitution) are usually employed in these studies. Chinese hamster ovary (CHO) and lung (V79) cells are most commonly used, because they grow rapidly and they have a high plating efficiency. In addition, the mutation frequency is high and the cells are smaller than human cells, so that many more can be cultured per plate. It should be noted that rearrangements in a number of the chromosomes have occurred during many years of cultivation of V79 and CHO cells and this may significantly affect the mutation frequency and metabolic activity, (Howard-Flanders, 1981). Syrian hamster kidney (BHK) cells have the advantage that they retain a greater capacity for enzymic activation of carcinogenic hydrocarbons than the V79 cells. Mouse lymphoma cells (L5174) are also used, because they are easily cultured and can be cloned in soft agar. For a summary of the various mammalian cell lines used to detect point mutations at multiple gene loci see Figure 8.

Mammalian cells have been coupled with metabolic activation systems similar to those occurring *in vivo*, (eg. the host mediated assay, liver cell homogenates, ie. S9).

Mutant mammalian cell phenotypes differ from wild type with respect to growth under limiting nutritional (biochemical or serological) or environmental (eg. temperature) conditions, or they are resistant to cytotoxic drugs. Therefore, the main groups of mutants are auxotrophs, conditional lethals and drug resistant types. The selection of cells resistant to purine analogues such as 8-azaguanine or 6-thioguanine forms the basis of the most widely used system for isolating mutants in mammalian cells.

Figure 8. Selection of drug-resistant phenotypes for detection of mutation in mammalian cells. (from Casto, 1981)

Cell source and type	Gene Locus	Selection drug
Hamster		
V79	HGPRT	AG, TG
	TK	BrdU
	Na^+-K-ATPase	OUA
CHO	HGPRT	AG, TG
	APRT	AA, DAP
	Na^+-K-ATPase	OUA
BHK21	TK	BrdU
Mouse		
L5178Y	HGPRT	AG, TG
	TK	BrdU, TFT
	NA^+-K-ATPase	OUA
Human		
Diploid fibroblast	HGPRT	AG, TG
	APRT	DAP
	NA^+-K-ATPase	OUA
Lymphoblasts	HGPRT	AG, TG

Key:
AG = 8-azaguanine
TG = 6-thioguanine
BrdU = bromodeoxyuridine
OUA = ouabain
Aa = 8-azaadenine
DAP = diaminopurine
FTF = trifluorothymidine

Resistance to these compounds is caused by a reduction in activity of hypoxanthine-guanine phosphoribosyltransferase (HGPRT), as a result of a mutation in the HGPRT locus on the X-chromosome. Bromodeoxyuridine (BrdU) or trifluorothymidine (TFT) which are phosphorylated by thymidine kinase (TK) may also be used to select for drug resistant mutants. In man, mutations at the HGPRT locus cause a genetic disease (the X-linked Lesch-Nyhan syndrome). Mutants will survive depending upon their ability to synthesise appropriate nucleotides by alternate metabolic pathways (Casto, 1981; Howard-Flanders, 1981).

Mutations affecting a membrane-associated sodium-potassium-ATPase produce resistance to ouabain (steroid). The spontaneous frequency of ouabain-resistant mutants in CHO cells is $\sim 10^{-6}$ per generation but this can be increased to $\sim 10^{-4}$ by treatment with ethyl methane sulphonate (EMS) and other potent mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Certain substances such as diphtheria toxin and trichodermin, which inhibit protein synthesis, may also be used to select for drug resistant mutants.

Mouse L5178Y and human lymphoblasts can be grown in suspension culture (i.e. mutant selection is in soft agar) unlike most other cell types, which are plated on solid agar surfaces. As with other tests a standard protocol has been developed for the mouse lymphoma test, which incorporates an S9 activation system, and it has been routinely used in screening chemicals, including hair dye components and the flame retardant, Tris (Hollstein et al., 1979).

The density of cells plated for mutant selection directly influences the survival and appearance of mutants for certain selective agents. Apparently, cell-to-cell communication can promote cross-feeding of nucleosides and their analogues from wild type to mutant cells. Drug-resistant cells may then be connected to drug-sensitive cells, by the passage of drug metabolites and the observed mutation rate. The type of cell will also affect the cell density at which such cooperation may occur and assays in agar will eliminate this problem.

Drug-resistant colonies are counted after the appropriate incubation period in selection medium and mutation frequencies for control and mutagen-treated cultures are computed (as a function of total viable cells exposed). For details of various assays and limited validation studies using different cell lines see Howard-Flanders (1981), and Hollstein et al. (1979).

The choice of cell system and genetic marker under investigation will considerably affect the duration of the test and expression times may also vary for the different mutations. Whilst these assays do provide some extra information, they are not as rapid as the microbial assays nor are they as well-validated, i.e. they could not be regarded as suitable alternatives for screening programmes. Anderson (1981) estimates that one person can test one compound over five doses every two months (allowing for repetition and difficulties). At this time there appears to be qualitative agreement between the most commonly adopted mammalian assays and the widely used S9 activated bacterial mutagenicity systems.

5.4 DNA Damage and its Subsequent Repair

Chemical carcinogens are known to cause various types of damage to DNA, e.g. single strand breaks, base alkylation and strand cross-linking. The events which follow such damage may be lethal, remain unhealed or repair may restore a normal genome, although misrepair will alter the gene (recall Section 2.3). Damage to DNA can best be measured using some parameter of DNA repair. See Figure 9 for some examples of repair mechanisms.

Studies of repair processes in mammalian cells have been based on bacterial studies of DNA repair. The major types of DNA damage lead to excision repair. This repair process occurs prior to replication and is usually error-free in most normal bacterial and mammalian cells (Anderson & Longstaff, 1981; Kouri, 1980).

Excision repair or "cut and patch" repair occurs when a chemical lesion that is recognised by endonuclease is excised by exonuclease. The damaged portion of DNA (which can range from as little as three nucleotides to one hundred nucleotides), is degraded and the missing portion resynthesised by polymerases that copy the intact strand and thereby reconstitute the original DNA strand. The newly synthesised portion is joined to the existing DNA by ligase. This repair process is termed error-free, because relatively few mistakes are made that are detected as mutations or transformational events.

DNA repair that takes place following semiconservative replication of damaged DNA is termed post-replication repair and may occur in both bacterial and mammalian cells (Kouri, 1980). Certain types of DNA damage such as pyrimidine dimers will disrupt the secondary structure of the double helix, but do not entirely block DNA replication. The lesion is by-passed in newly synthesised DNA, but a gap may be left in the daughter strand opposite the damaged site. This gap is sealed in the post-replication repair process in bacteria in a way that is unknown in mammalian cells (Anderson & Longstaff, 1981). Post-replication

Figure 9. Examples of some DNA repair activities induced by chemical mutagens (from Brusick, 1980)

Excision repair	SOS repair
Damaged DNA areas excised and new sequences resynthesized in gaps	Appears to be confined to prokaryotic organisms such as bacteria; has little or no effect in human DNA repair
Multiple enzymatic functions involved in repair, including recognition of damaged sites, nuclease base removal, polymerase base replacement, and ligase reattachment of DNA ends	The repair system is not normally present, but is induced by DNA damage that cannot be repaired by the normal excision or postreplication repair processes
Involved in human DNA repair	Often leads to error-prone repair among surviving cells
Broad range of DNA lesions repaired, including altered bases, larger lesions or cross-linking of DNA	

repair is significant in rodents and is believed to be of potential importance in man. This repair process tends to be error-prone.

Recombination plays an important part in post-replication repair of bacterial cell DNA damage, but its role has not been demonstrated conclusively in mammalian cells (Kouri, 1980).

Another repair mechanism that appears to be confined to bacteria is S.O.S. repair. Repair activity is induced by exposure of cells to mutagenic chemicals (and also UV). See Figure 9.

Individual organisms will vary in their susceptibility to genotoxic agents, as a result of differences in their repair capacities. In theory, DNA repair deficiencies or the inhibition of DNA repair could lead to increased susceptibility to chemical carcinogenesis (Kouri, 1980).

Primary repair mechanisms depend upon functional enzymes, which can be saturated by DNA lesions caused by high test chemical concentrations and therefore many lesions are fixed as mutations or cause chromosome damage. At low dose levels, repair processes may significantly modify the risk of exposure to chemical mutagens.

A number of tests are available for the detection of DNA repair phenomena. These tests include differential inhibition or killing in bacterial strains with and without repair processes and gene conversion in yeast strains (see Sections 5.1 and 5.2 respectively). Other methods of measuring DNA repair include sister chromatid exchanges in mammalian cells (see Section 5.5.1), or measurement of unscheduled DNA synthesis (UDS), resulting from DNA excision repair.

5.4.1 Unscheduled DNA synthesis (UDS)

Autoradiographic measurement of UDS is currently the most frequently used technique for assessing repair synthesis (IARC/WHO, 1980).

A range of human cells (e.g. lymphocytes, fibroblasts) or rodent cells such as mouse lymphoma L5178Y, rat primary hepatocytes and hamster kidney BHK21 can be used in tests to measure UDS. The cells are exposed to the test chemical in the presence of radioactively labelled thymidine, and the incorporation of the tritiated thymidine into repaired DNA is measured. Hydroxyurea is used to suppress semiconservative or normal DNA replication in this technique, because it allows the detection of excision repair which would otherwise be masked. However, hydroxyurea suppression of replicative DNA synthesis is subject to interference from other chemicals that restore the normal replicative synthesis. This effect could be interpreted as normal repair (IARC/WHO, 1980; Anderson & Longstaff, 1981). To overcome this, arginine starvation in human fibroblasts inhibits normal DNA replication, so that DNA repair may be readily detected. Certain cell types (e.g. rat primary hepatocytes) are essentially non-dividing and therefore UDS can be measured in these cells without external control of DNA synthesis (Hollstein et al., 1979). Autoradiography is the preferred measurement technique, although liquid scintillation counting may also be used, but occasionally residual DNA replication can produce high background counts in the latter technique.

Although the extent of UDS in human cells is greater than that obtained in rodent cells after treatment with a number of carcinogens, rodent cells (in particular, rat primary hepatocytes) provide one of the most efficient methods for detecting UDS. Rodent cells retain many metabolic activating enzymes, which enhance the detection of a number of carcinogens. Several types of chemicals have been tested in rodent systems and various direct-acting carcinogens as well as those requiring metabolic activation have been detected (e.g. aromatic amines, nitrosamines, aflatoxin). However, more than one hundred chemicals have been screened for carcinogenicity in other tests using human diploid fibroblasts, which retain few microsomal activating enzymes. Not surprisingly, many carcinogens that require

activation produced negative results (e.g. benzo(a)pyrene, aromatic amines). This test has improved with the addition of S9 mix and weak activity has been detected for agents such as aflatoxin B_1 (Hollstein et al., 1979).

5.4.2 DNA fragmentation

DNA damage may also be measured by detecting single strand breaks that may occur either as a direct result of the interaction of a chemical with DNA or the activity of DNA repair enzymes in attempting to restore the distortion in the molecule. Although many mutagens cause DNA strand breaks, such breaks may also arise as a result of non-specific toxic effects. The size of single strand DNA can be measured by the sensitive technique of sucrose gradient centrifugation or by an alkaline elution technique, which is also relatively simple and rapid (IARC/WHO, 1980).

Cultured cells (e.g. Chinese hamster V79) have been used in alkaline elution assays. After exposure of the cells to labelled thymidine and the test chemical, DNA fragmentation may be determined by increased alkaline elution of labelled DNA from cells lysed (by addition of anionic detergent at pH 10) on a membrane filter. The rate of elution is a function of the molecular weight of the eluted DNA and the intact DNA is retained on the filter. An alternative to measuring the DNA by radioactivity is provided by fluorimetric assay.

S9 activation has been incorporated into this test and several carcinogens and non-carcinogens have been tested, but the system has not been sufficiently validated (Hollstein et al., 1979; IARC/WHO, 1980).

5.5 Chromosome Damage

Chromosome abnormalities have been observed in malignant cells from cancerous tumours in both animals and man. In fact, a number of 'chromosome breakage' syndromes are known to be associated with increased incidence of cancer. In some cases, it is still unclear whether or not chromosome aberrations occur as a result of tumour development or whether they contribute directly to the cause of cancer (Evans et al., Report 8, IARC/WHO, 1980).

Damage to chromosomes is characterised by changes in the number or structure of chromosomes following exposure of dividing cells to chemical mutagens. It should be noted that chromosome damage can also arise from an irregular event during segregation of chromosomes at mitosis (somatic cell division).

Cytological methods developed from research into chromosome damage caused by ionizing radiation may be used to detect chromosome aberrations caused by chemical mutagens. The variations in numbers of chromosomes are the easiest to observe. For example, aneuploid conditions are recognised by variation in the number of single chromosomes within a set (recall Down syndrome or trisomy 21). Euploidy describes conditions in which there is a variation of the entire set of chromosomes.

Structural changes such as breaks may be observed both in the whole chromosome (i.e., paired chromatids) or a single chromatid arm. Achromatic lesions or "gaps" sometimes appear, but their origin and clinical significance is unknown. Dicentric chromosomes may appear following exchanges between two chromosomes resulting in a chromosome with two centromeres and a fragment. All of the above structural alterations, along with acentric fragments and ring chromosomes are non-transmissible because effects may be lethal, whereas inversions of parts of the chromosome, reciprocal translocations and deletions are sometimes stable structural alterations that can be transmitted to the progeny (Anderson & Longstaff, 1981; Evans et al., Report 8, IARC/WHO, 1980; Natarajan & Obe, 1980).

The background level for chromosomal aberrations is variable and therefore small changes in aberrations such as those resulting from exposure to low doses of chemicals may be difficult to determine. From a statistical viewpoint the lower the background level for chromosomal aberrations, the more difficult it is to show small differences, because many more samples must be processed. This technique then becomes time-consuming and expensive. Fortunately, this does not apply to all cytogenetic analyses; for example, the detection of sister chromatid exchanges (see Section 5.5.1) is not subject to the same spontaneous background variability and a positive response can be observed at a lower concentration of mutagen (Brusick, 1980).

The majority of chemical mutagens produce chromosome aberrations, which develop as a result of DNA lesions in the chromosome that are subject to cellular repair and misrepair and are therefore translated into breakage and exchange events during cell replication. (Recall that not all mutagens are clastogenic agents.) The type of clastogen and the stage of the cell cycle at which the target cell was exposed both influence the type of aberration produced (i.e. chromosome or chromatid). The induction of chromatid type alterations is less dependent upon the cell cycle stage, but cells are usually examined during the first mitosis following exposure. The most effective clastogenic agents currently recognised are alkylating agents, which are also good inter- and intrastrand cross-linking agents, such as nitrogen mustard and triethylene melamine (TEM). The observation of such induced chromosomal aberrations is delayed, because the chromosomes must pass through a replication phase before they are visualized in the second mitosis following exposure (Natarajan & Obe, 1980).

Cell preparations are scanned for metaphase spreads because it is at this stage of mitosis (somatic cell division) that

chromosome and chromatid aberrations are most easily detected. Aberrations at metaphase have been compared with those at anaphase and it appears that the detection and scoring of fragments is reduced by fifty percent at anaphase and that bridges are also observed at a lower frequency. The scoring of fragments and bridges in anaphase cells represents a limited proportion of possible chromosome and chromatid aberrations, but it is less time-consuming and the end points are readily observed (Evans et al., Report 8, IARC/WHO, 1980).

5.5.1 The Sister Chromatid Exchange (SCE) Test

During replication of DNA containing a chemically induced lesion, exchanges may occur between the two daughter DNA strands. These molecular exchanges are expressed at the cytological level as exchanges between the two sister chromatids of a metaphase chromosome (Wolff in: Stich & San, 1981). The increased incidence of SCE are considered to be a reflection of DNA repair during replication (Natarajan & Obe, 1980).

SCEs do not produce any change in chromosome morphology, because they involve reciprocal (equal symmetrical) exchange at homologous loci between sister chromatids. Early differentiation of sister chromatids was achieved by using an autoradiographic technique (incorporation of tritiated thymidine), but results were not altogether satisfactory (Wolff in: Stich & San, 1981). Current methods rely upon differential staining techniques. Cells which have passed through two cycles of DNA replication in the presence of 5-bromodeoxyuridine (5-BrdU) have chromosomes with one chromatid unifilarly substituted and the other bifilarly substituted. Alternatively, for one round of DNA replication with 5-BrdU, chromosomes are produced with one unsubstituted chromatid and the other singly substituted. The difference in chemical constitution of the sister chromatids is detected by staining with Giemsa, fluorescent dye or a combination of both (Wolff, 1981). SCEs appear as light-dark exchanges between chromatids. Such exchanges are induced by 5-BrdU alone,

but their frequency is greatly increased on exposure to chemical mutagens.

A major advantage of this rapid and relatively easy cytogenetic method is that lower concentrations of mutagen will produce a positive response. In addition the analysis can be performed in many higher eukaryotes. Most studies on the chemical induction of SCEs in vitro have used Chinese hamster ovary (CHO) cells or human peripheral blood lymphocytes. The former have been particularly useful, because they have a short generation time (12-14 hours) and a low chromosome number (20-22) in comparison with the human (46) and mouse (40). Direct acting compounds such as alkylating agents are readily detected, but compounds that require metabolic activation are also detected. Positive results in this test generally indicate that a compound is a mutagenic carcinogen, i.e. few false positive results are generated (Latt et al., 1981). The greatest advantage from the point of view of occupational health and safety lies in its potential value as a test for monitoring an exposed workforce.

Unfortunately, the exact molecular basis of SCE and the relationship between SCE formation, DNA repair and mutagenesis has not yet been determined. Whilst SCE is considered to be an indirect measure of DNA damage, the extent of the damage may vary greatly from one agent to another. Response to compounds such as bleomycin that are capable of inducing double-strand DNA breaks is poor. False negatives may arise from inadequate activation or chemical reactivity before the target is reached. Certain non-mutagenic carcinogens such as diethylstilboestrol may be negative in SCE analysis (Latt et al., 1981).

Natarajan and van Kesteren-van Leeuwen (1981) recommended the use of SCE and chromosome aberration tests in combination for prescreening carcinogenic mutagens. Positive results were obtained in both tests for seven compounds (twenty were tested) and certain compounds were positive in the chromosome aberration

test only, e.g. diethylstilboestrol and hydrazine sulphate. Other chemicals such as 1-naphthylamine and 4-dimethylaminoazobenzene produced weak positive results in the SCE tests (Ashby & de Serres, 1981).

Perry and Thomson (1981) point out that the SCE test is not well validated at this time and there is no universal agreement concerning the significant level of SCE incidence for test purposes. The toxicity of chemicals that reduce the population of dividing cells and thereby induce a low frequency of SCE must also be considered (e.g. benzidine). Perry and Thomson (1981) concluded that a 50% increase over the control SCE level plus a dose response would be an acceptable end point for the SCE test. (For details see Ashby & de Serres, 1981.)

5.5.2 The Micronucleus Test : in vitro

Micronuclei are formed by broken chromosome or chromatid fragments which are left behind during anaphase movement of chromosomes in mitosis. Only a limited proportion (about 10%) of chromosome aberrations give rise to micronuclei, but scoring is relatively simple, as they can be observed during interphase (i.e., persist throughout the whole cell cycle). Therefore a large proportion of a cell population may be analysed.

Chromosomal aberrations are usually scored at the first mitosis following exposure, whereas SCE are scored at the second mitosis. The easily detected aberrations at the first mitosis are mainly cell lethal events that require early scoring.

Recent work by Heddle (1981) has combined the SCE analysis and the Micronucleus test, because both effects can be scored on the same SCE cytological preparation. The highest incidence of micronuclei has been demonstrated (in X-irradiated human lymphocytes) between the third and fourth mitoses, after which they are diluted out through cell division. Scanning at second mitosis will not produce the maximum scoring, but this is compensated by greater reliability, because absence of micronuclei

cannot be associated with failure of cell division, but relates directly to a lack of chromosomal aberrations.

Generally, the SCE and Micronucleus tests have similar sensitivities and an increase in SCE is usually accompanied by a similar increase in micronuclei. However, the converse does not always hold true (Heddle, 1981).

This test has not been widely used and recent data regarding the statistics of micronucleus production have not yet been published. Positive responses have been based on the demonstration of reproducible dose response curves. As with the SCE test, the micronucleus test is not well validated at this time. With improved protocols and a wider data base, the test has some potential and along with SCE analysis it may become a more useful test to demonstrate occupational exposure.

5.6 Cell Transformation

The precise type of genetic change that is the major cause of chemical carcinogenesis in man is unknown. Other molecular changes are thought to occur following the initial genomic change (mutation), in order for the cell to acquire transformation properties, i.e. non-mutagenic steps of promotion and fixation that permit expression of the DNA lesion. Therefore, the development of *in vitro* test systems, which more closely reflect neoplastic transformation *in vivo* are of great value in defining the carcinogenic process. A number of transformation systems have used mammalian cell lines (e.g. mouse, rat, hamster) and now tests using human cells are under development. In this way, differences in sensitivity to transformation between human and other mammalian cell lines can be observed.

There are four different methods of assaying the cell transformation capacity of a chemical, i.e. passage of cells in mass culture, clonal transformation, formation of transformed cell foci, and growth in soft agar (Casto, 1981).

5.6.1 Mass culture

The mass culture technique uses cells that have undergone successive growth in tissue culture. These cells are dosed either continuously or acutely with a test chemical and observed for changes in morphology and growth patterns. Such changes are compared with untreated control cells showing stable morphology. A range of cultural differences have been recorded such as increased glycolysis, growth at increased serum concentration and loss of oriented cell growth patterns. With further development the cells are able to grow in soft agar and they will also form tumours in susceptible hosts.

The large number of cells with potential for transformation and the amplification provided by successive culture passages

are advantageous, especially for the detection of chemicals that transform at low frequency. The time lapse between treatment and observed effect is a major limitation along with the lack of quantitative data linking a test chemical with effect on the cell (Casto, 1981).

5.6.2 Clonal assays

Colony techniques require culture plating at cell densities of $\sim 10^5$/plate and incubation overnight, before the addition of test chemical for a set period or the duration of the experiment. Alternatively, a cell monolayer may be exposed to the chemical, then cells trypsinized and plated as above.

Following eight to ten days incubation, the colonies are counted and neoplastic transformation recorded as the proportion of transformed colonies in a test population. Chemically transformed cells form dense colonies. These cells tend to aggregate in heaps and show criss-crossing of fibroblasts within colonies. Normal, untreated cells do not grow in layers. They appear in parallel arrangements and colony margins are clear and even.

This method has a number of advantages, for example, survival and transformation are assayed on the same cell population and therefore the results are quantitative. In addition, the time between treatment and observed effect is often less than ten days, and transformed cells are easily scored. The reduced time period to observed effects also eliminates cell passage.

Difficulties associated with this method include comparison with control colonies, which must be uniform in appearance. Also the cell lines used (e.g. secondary hamster embryo cells) may be difficult to clone and the recognition of chemically transformed colonies needs expertise and experience. Assay cultures must be composed of cells, which on cloning, produce colonies of cells containing typically small, elongated fibroblasts (i.e. cultures producing colonies consisting of epithelial cells/fibroblasts with decreased width:length ratios should not be scored).

5.6.3 Focus assays

These assays utilise early passage mouse cell lines (e.g. BALB/c 3T3) and hamster embryo cultures. Transformed cell foci are observed against a background monolayer of untransformed cells. Cultures are plated out at 10^3 - 10^4 cell numbers and following overnight incubation the test chemical is added. Chemical exposure usually ranges from one to six days, after which fresh medium is added at regular intervals. At the three to four week stage transformed cell foci may be detected in hamster and mouse embryo cells and at six weeks, similar foci are observed in C3H10T½ mouse cells. Transformed cell foci are recognised as darkly staining dense cell masses that appear to invade the surrounding cell monolayer. Analysis using a stereomicroscope reveals that most of the transformed foci are heaps of fibroblast-like cells which criss-cross or a dense patch of non-polar cells with randomly distributed fibroblasts at the edges. It is important to carefully examine the dense areas, in order to differentiate transformed foci from treated cells piled in parallel alignment.

This assay is less time-consuming (cf. colony assay), because only grossly visible dense areas require microscopic analysis. In addition, neither a uniform cell morphology nor strict cultural conditions are critical to this assay.

The twenty-five to thirty day period required for optimum development of foci constitutes the major disadvantage of this assay. Aggregates of cells produced by the toxicity of test chemicals must also be examined to avoid their inclusion as positive results. A similar degree of expertise and experience to that required for scoring positive results in the clonal assay, is required here also.

5.6.4 Soft agar assays

Transformation in cell lines of Chinese or Syrian hamsters treated with chemicals is recognised by the ability to produce colonies in soft agar. The chemical is added to cells in suspension for twenty to sixty minutes, cells are washed, and then

plated into a soft agar dissolved in cell culture medium. The cells in soft agar are scored as transformed, because untreated cells rarely form colonies. If 10^3 cells from such colonies are implanted in hamsters, tumours are produced.

The use of cell suspensions may facilitate the detection of those chemicals with a short half-life. This method is easy and rapid and may be adapted to include the S9 metabolic activation system. Such readily available clones provide uniform populations for examination.

5.6.5. Application of transformation assays in screening programmes

Although transformation assays are still very much in the development stage compared with some of the other short-term *in vitro* tests (e.g. bacterial mutagenicity tests), their value in detecting promoters such as pharbol esters and alkanes has already been recognised. Certain toxic chemicals such as hydrazine derivatives, steroid hormones, chlorinated hydrocarbons and asbestos go undetected in routine microbial assays, but they are detected in selected mammalian assays (Casto, 1981).

For the purposes of routine screening of chemicals to detect potential carcinogens the rapid and relatively simple soft agar method, which incorporates the S9 activation system, is most attractive. The capabilities of focus assays using Syrian hamster embryo (SHE) cell lines or mouse (BALB/c 3T3) cell lines have also expanded as a result of incorporating primary hepatocytes for activation, (Brusick, 1980).

A validation study undertaken by Purchase et al., (1978) using the BHK cell line with S9 activation showed that fifty-three of fifty-eight carcinogens were positive and sixty of sixty-two non-carcinogens were negative. More recently, styles (1981) used the BHK cell line, which has a low frequency of spontaneous transformation, in a smaller chemical screening study. Again, growth in soft agar was the indicator of transformation and twenty-two of

twenty-five carcinogens were positive and twelve of seventeen non-carcinogens were negative. Differences in serum/agar constitution were found to modify the end point of the test (i.e. phenotypic transformation was influenced by charge and viscosity). It is clear that rigid protocols and considerable technical expertise are required if accurate and consistent results are to be obtained from transformation assays. To date, it has been difficult to use such tests for routine screening purposes. Daniel and Dehnel (1981) highlighted some of the problems of the soft agar method using BHK 21 cells. Factors which were found to critically affect the test results included choice of serum to improve cell plating efficiency (e.g. new born calf or foetal) for mammalian cell culture and shaking during incubation of cells with test chemical. Daniel and Dehnel (1981) recorded sixteen of twenty-three carcinogens positive and only three of fourteen non-carcinogens negative. Therefore, they considered that the low specificity of the test reduced its value in terms of routine screening, at this time. Verification from other workers would help in determining the degree of specificity. However, these tests do provide some useful additional information (see review by Hollstein et al., 1979, for further details of transformation assays). The current methods of assay coupled with the cell lines used in these systems are summarized in Figure 10.

Figure 10. Summary of transformation assays for screening chemical carcinogens *in vitro*. (from Casto, 1981).

Established *in vitro* systems:	Assay method
1. Syrian hamster embryo (SHE) cells	colony, focus, mass culture
2. Mouse prostate C3H cell line	focus
3. Mouse embryo 10T½ cell line	focus
4. Mouse BALB/3T3 cell line	focus, colony
5. Guinea pig embryo cells	mass culture
6. Rat embryo cells (Fill & F1706)	focus, mass culture

in vitro systems being developed:

1. BHK21 cell lines
2. Epithelial cells
   a. mouse and rat epidermis
   b. rat foetal lung
   c. epithelial-like cells from organs and tissues (liver, bladder, trachea, salivary gland, submandibular gland
3. Human cells
   a. osteosarcoma
   b. neurofibrosarcoma
   c. diploid skin fibroblasts

6. ADVANTAGES AND LIMITATIONS OF THE TESTS FOR SCREENING PURPOSES

Many of the advantages and disadvantages of the individual tests have been discussed in the earlier sections. However, it is worth summarizing some of the major points, particularly with respect to the interpretation of anomalous results.

False negative responses may be obtained for a number of different reasons. The most obvious reason for a negative result in an *in vitro* test is that the carcinogen may elicit its effect through a non-mutagenic mechanism (e.g. hormones or physical carcinogens such as asbestos). However, inadequate metabolic activation systems are a more common cause of false negative results. It should be noted that *in vitro* procedures are unable to detect promutagens which are activated by routes other than the liver, e.g. 1,2-dimethyl hydrazine, cyclamates and citrus red dye 2 have been reported to be metabolised by the intestinal flora (legator & Rinkus, 1981). Therefore, the activation system should be optimised for each *in vitro* test system, although some flexibility with respect to activation is also desirable (see Fluctuation Test 5.1.6). It is also essential to make the correct choice of *in vitro* assays for the particular class of chemical under examination. The importance of using a battery of short-term tests is emphasised by the fact that almost all the carcinogens which produce negative results in the *Salmonella*/microsome test have been reported positive in at least one other short-term test (e.g. acetamide and hydrazine derivatives detected in mammalian cell mutagenicity assays).

False positive results require equally careful assessment. The non-carcinogenic status of a chemical may have been inadequately defined (i.e. questionable results from long-term animal studies). Also, *in vitro* tests cannot exactly reproduce the *in vivo* metabolic pathways (e.g. detoxification processes), nor can they simulate whole body chemical transport effects. Finally, an *in vitro* assay may respond to a physical or chemical property of a test compound, which may not be carcinogenic (i.e., positive mutagen, negative carcinogen).

Well defined test protocols are essential to the rapid production of reliable and reproducible results (see Appendix I for an example test protocol). The rapid, simple techniques are accurate descriptions, always provided that qualified, experienced workers undertake these tests. Considerable variability in results produced by workers in the same and different laboratories emphasises this point.

6.1 Monitoring Industrial Exposure

From the occupational standpoint, there are two approaches to assess the effects of environmental agents on exposed workers. The presence of mutagens in the occupational setting may be detected by monitoring the environment in which the worker is exposed. This is relatively inexpensive and requires limited time and equipment. An alternative approach of assessing health effects to the exposed workforce is biological monitoring. This method takes into account individual differences in the exposed population and provides more detailed data on the fate of the chemical in the body and hence the potential for toxic manifestations. However, this method can be more complex and time-consuming.

The Salmonella/microsome assay (Ames test) is one of the most useful short-term tests for detecting potential carcinogens in complex mixtures. For example, results from such tests facilitated the isolation and identification of active agents in extracts of environmental particulates collected within an Ontario steel foundry (McCalla et al., 1981). Generally, linear dose response curves are obtained for a reasonable range of mutagen concentrations and therefore the tests provide a quantitative assessment of the mutagenic potency of the substance under examination.

Cytogenetic analysis is a well established procedure for detecting chromosomal aberrations and therefore it is widely used to evaluate the adverse effects of industrial exposure on

man (Legator, 1981; Mendelsohn, 1981). Scoring of chromosome aberrations requires considerable expertise, is time-consuming and costly (see section 5.5). Although automatic scanning techniques can be used to facilitate karyotyping, the analysis of complex chromosome aberrations is still too complicated for the computer. One of the most promising tests undergoing development at this time is the Sister Chromatid Exchange (SCE) test, but careful evaluation in extensive pilot study programmes in the occupational setting is essential before it can be routinely applied. Numerous lifestyle factors, such as smoking must be taken into account (Sorsa et al., 1982). Successful pilot studies have been conducted on small populations such as cancer patients receiving antineoplastic drugs (e.g. cyclophosphamide) where a positive correlation between exposure and SCE was found. Studies are now being extended to nursing staff handling these drugs. Occupational exposure has also been demonstrated in other studies, for example the increased incidence of SCEs in a population of exposed petroleum workers (Brusick, 1980).

In addition to methods used for evidence of direct damage in human somatic or germ cells (i.e. chromosome analysis, sperm analysis), secondary analyses such as screening urine or faeces for excreted mutagens can be undertaken. Usually, a non-human indicator organism (e.g. Salmonella) is employed in these tests (Bora et al., 1982).

Urine analysis is currently the fashionable choice for biological monitoring, because this technique is easily applied and it is reasonably cost and time effective. In addition, urine analysis is fairly well documented and some indication of background levels of mutation can be estimated. Much of the background information has arisen from studies on humans exposed to various chemotherapeutic drugs, cigarette smoke and industrial chemicals. A few pilot studies have involved workers in plants producing styrene, rubber plant workers and coke plant operators. However, results from such occupational monitoring

studies have varied for a number of different reasons. For example, sample timing is critical, because substances rapidly eliminated from the body may go undetected and some chemicals will not be converted to mutagenic metabolites that accumulate in the urine. It is clear that considerably more research work is required before this technique can be applied as a routine monitoring method for occupational populations (Bora et al., 1982).

A number of variables affect all human monitoring techniques. These include lifestyle factors such as smoking, alcohol consumption, drug usage, work habits (i.e. use of protective equipment) and genetic predisposition. The limitations of individual tests and the variables which affect them need to be specified at the beginning of any monitoring programme and the design developed to take these into account.

A set of techniques which meet all the ideal requirements of a system for monitoring human populations with respect to genetic damage, is not currently available. However, at this time, some very useful information may be obtained by the careful selection and use of short-term tests. Continued research will likely provide greatly improved test systems.

7. SUMMARY AND CONCLUSIONS

A great variety of *in vitro* tests currently exist for the detection of potential mutagens and carcinogens, but each test has its own individual advantages and limitations and relatively few are sufficiently well-validated at this time for routine use.

Recall that chemical mutagens are defined as substances that may cause inherited mutation in man leading to genetic disease and by empirical correlation have the potential for causing cancer. No single short-term test will detect all mutagens, but a reasonably good judgement can be formed about the potential carcinogenicity of a chemical to man based on the results from several carefully selected short-term mutagenicity tests. Positive results in more than one well-validated mutagenicity test system increase the confidence in the designation of a chemical as a potential carcinogen. Positive results obtained in only one test system cannot be disregarded, but merely interpreted as less predictive of potential carcinogenic activity in man. As previously mentioned, the most useful approach to screening for mutations is to set up batteries of tests that not only cover a number of genetic end points, but also incorporate an element of specific end-point redundancy. Generally, individual tests are chosen to detect specific genetic end points such as gene mutation, chromosomal effects or primary DNA damage. The advantage of combining several of these individual tests into a battery is to minimize the number of genotoxic agents that may escape detection because of the technical limitations of certain tests.

Strict protocols are a necessary requirement for the standardisation of short-term mutagenicity tests that could be used by regulatory authorities in making decisions regarding the potential health hazard from exposure to certain chemicals. However, overly stringent protocols would exclude the detection of certain chemicals. For example, dialkylnitrosamines require preincubation at relatively high test chemical exposure concentrations with a mammalian activation system, to induce positive mutagenicity in bacterial tests. Therefore, in making regulatory decisions the chemical, biochemical and

pharmacodynamic properties of a substance should be taken into consideration, in order that the correct *in vitro* tests are chosen.

The most convincing evidence supporting the human carcinogenicity of a given chemical comes from scientifically acceptable epidemiological studies of occupationally exposed workers that show a significant increase in the incidence of cancer over normal background levels. In the absence of such positive findings and if data from long-term animal studies is questionable, then the role of short-term *in vitro* tests becomes more important in predicting the carcinogenic potential of chemicals.

From the above discussion it should be clear that the following conclusions can be drawn regarding the value of *in vitro* tests for evaluating the carcinogenic potential of a given chemical to man.

1) *In vitro* tests are valuable tools in establishing the mutagenicity of a given chemical or mixture of chemicals. The fact that a chemical is detected as a positive mutagen is suggestive evidence, although not conclusive proof, of its carcinogenic potential, because many of the known chemical carcinogens are mutagens.

2) It is essential to select a group of tests that are capable of detecting the range of possible genetic end points in order to firmly establish positive results for a range of compounds.

3) Short-term tests can aid in the regulatory decision-making process regarding the potential genetic consequences of exposure to a given chemical. However, this does not provide an accurate indication of risk to man associated with specific chemical exposure. Before risk can be estimated, the appropriate *in vivo* data must be available.

8. RECOMMENDATIONS

Based on the foregoing information, the following recommendations are made with respect to the use of short-term mutagenicity test results for the purpose of evaluating the carcinogenic potential of a given chemical to man.

1) Battery systems also incorporate short-term *in vivo* tests and therefore it is logical to evaluate the status of such tests in a similar way to this report, before a valid estimation of the risk to man of chemical exposure can be obtained.

2) The whole field of short-term testing is one of rapid development, in that many tests are currently undergoing validation studies and improvements (e.g. of activation systems). Therefore, it is essential that qualified professionals maintain an active awareness of developments in both *in vitro* and *in vivo* testing, in order to intelligently guide the interpretation and application of such data in the formulation of occupational exposure standards, regulations, guidelines or policies by Government agencies.

3) There is a definite need to further develop those *in vitro* tests, which lend themselves to routine monitoring of occupational populations for the purpose of detecting adverse effects from exposure to the wide variety of industrial chemicals. Specific test development can best be achieved by close liaison with well recognised research workers in the field.

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APPENDIX I

(from Brusick, 1980)

AMES SALMONELLA/MICROSOME PLATE ASSAY

The objective of this study is to evaluate a test article for mutagenic activity in a bacterial assay with and without a mammalian S9 activation system.

Materials

The following microbial strains of S. typhimurium should be routinely used in the plate test:

Strain designation	Gene affected	Additional mutations			Mutation type detected
		Repair	LPS	R Factor	
TA-1535	hisG	ΔuvrB	rfa	--	Base-pair substitution
TA-1537	hisC	ΔuvrB	rfa	--	Frameshift
TA-1538	hisD	ΔuvrB	rfa	--	Frameshift
TA-98	hisD	ΔuvrB	rfa	pKM101	Frameshift
TA-100	hisG	ΔuvrB	rfa	pKM101	Base-pair substitution

The Salmonella cultures can be obtained from Dr. B. N. Ames University of California at Berkeley.

Each strain should be cultured in nutrient broth for approximately 16 hr at 37°C (titer of 10^8-10^9 cells/ml) and used for the mutagenicity test. Examine the master culture of each strain for appropriate genetic markers (his, uvrB, rfa, pkM101). The data of culture preparation should be documented and the daily cultures should be examined periodically for appropriate genetic markers as described by Ames, et al. (1975).

An S9 homogenate should be used as the activation system (see Appendix A). The 9000g supernatant is prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 (described by Ames et al., 1975). The S9 mix should have the following components:

Components	Concentration/ml S9 mix
NADP (sodium salt)	4 µmol
D-Glucose-6-phosphate	5 µmol
$MgCL_2$	8 µmol
KCl	33 µmol
Sodium phosphate buffer, pH 7.4	100 µmol
Organ homogenate from rat liver (S9 fraction)	100 µl

The bacterial strains should be cultured in Oxoid Media #2 (Nutrient Broth), with selective medium Vogel Bonner Medium E with 2% glucose. According to the methods of Ames et al. (1975), the overlay agar should consist of 0.6% purified agar with 0.05 mM histidine, 0.05 mM biotin, and 0.1 M NaCl.

Test Procedures

Prepare and label an appropriate number of tubes of molten overlay agar as well as Vogel Bonner plates.

To set up the stock solutions, the test article should be weighed or measured and diluted into a solvent. The test article and other components should be added to the overlay and the contents poured on the surfaces of the appropriate Vogel Bonner plates (see Figure 9.1).

The plates (once the overlay has solidified) should be incubated at 37°C for 36-72 hr and then scored for numbers of revertants per plate.

All tests should be evaluated using a minimum of five concentrations. In the standard plate test, at least seven dose levels of the test article, dissolved in a suitable solvent, should be added to the test system. This step allows for high dose toxicity without loss of the entire test. One to three plates per dose per strain should be used in the standard assay.

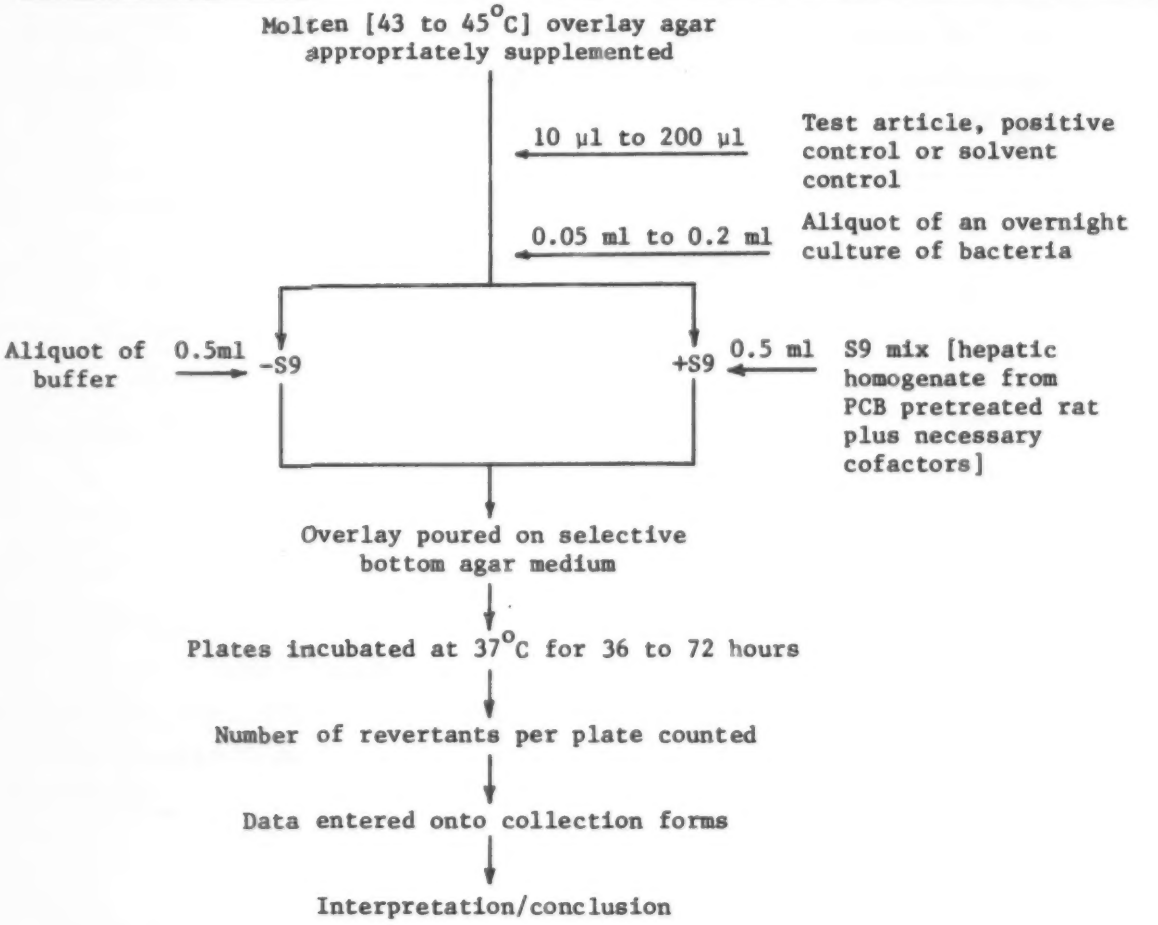
Molten [43 to 45°C] overlay agar appropriately supplemented
10 µl to 200 µl
Test article, positive control or solvent control
0.05 ml to 0.2 ml
Aliquot of an overnight culture of bacteria
Aliquot of buffer
0.5ml
-S9
+S9
0.5 ml
S9 mix [hepatic homogenate from PCB pretreated rat plus necessary cofactors]
Overlay poured on selective bottom agar medium
Plates incubated at 37°C for 36 to 72 hours
Number of revertants per plate counted
Data entered onto collection forms
Interpretation/conclusion

FIGURE 9.1. Reverse Mutation Assay in Bacteria

The standard test doses should be 0.005, 0.01, 0.1, 1.0, 5.0, and 10.0 µl per plate for liquids; and 0.5, 1.0, 10.0, 100.0, 500.0 and 1000.0 µg per plate for solids. Additional lower doses should be employed in the tests if toxicity is observed at the three highest doses. When no toxicity is observed, additional concentrations may be employed up to 50 µl or 5000 µg per plate. Alternatively to a preset concentration range one can elect to perform range-finding tests. A preliminary toxicity range-finding test with one strain such as TA-100 may be helpful in establishing a correct range of concentrations. The LC50 is appropriate as a high dose based on a toxicity test.

The articles that should be used as positive control agents are:

Assay			Concentration per plate (μg)	Responsive Salmonella strains
Nonactivation	Sodium azide	Water	1	TA-1535, TA-100
	2-nitrofluorene (NF)	Dimethyl-sulfoxide	10	TA-1538, TA-98
	9-aminoacridine (9AA)	Ethanol	50	TA-1537
Activation	2-anthramine (ANTH)	Dimethyl-sulfoxide	2.5	For all strains

Evaluation Criteria

Since statistical methods are not currently used, evaluation can be based on the criteria included in this protocol.

Plate test data will consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test article and the cells are incubated in the overlay for 36-72 hr and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

1. The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than non-replicating DNA.
2. The combined incubation of the test article and the cells in the overlay permits constant exposure of the indicator cells for 36-72 hr.

Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test article, the surviving populations on the treatment plates are essentially the same as that on the negative control plate. At high concentrations,

the surviving populations are usually reduced by some fraction. This protocol normally employs several doses ranging over two or three log concentrations; the highest of these doses is selected to show slight toxicity as determined by subjective criteria such as a slight reduction in the number of background revertants per plate.

Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test article may kill any mutants that are induced, and thus will not appear to be mutagenic.

Control Tests

Positive and negative control assays should be conducted with each experiment and should consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls should consist of the test article solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data can be compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

Evaluation Criteria for Ames Assay

Because the procedures to be used to evaluate the mutagenicity of the test article are semiquantitative, the criteria used to determine positive effects are inherently subjective and based primarily on a historical data base. Most data sets should be evaluated using the following criteria.

1. Strains TA-1535, TA-1537, and TA-1538. If the solvent control value is within the normal range, a test article that produces a positive dose response over three concentrations, with the highest increase equal to three times the solvent control value, should be considered mutagenic.

2. Strains TA-98 and TA-100. If the solvent control value is within the normal range, a test article that produces a positive dose response over three concentrations, with the highest increase equal to twice the solvent control value for TA-98 and TA-100, should be considered mutagenic.

3. Pattern. Because TA-1535 and TA-100 are derived from the same parental strain (G-46) and because TA-1538 and TA-98 are derived from the same parental strain (D3052), to some extent there is a built-in redundancy in the microbial assay. In general, the two strains of a set respond to the same mutagen, and such a pattern is sought. Generally, if a strain responds to a mutagen in nonactivation tests, it should do so in activation tests.

4. Reproducibility. If a test article produces a response in a single test that cannot be reproduced in additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria can be applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. A normal, spontaneous background range for a laboratory should be documented and compared to published ranges.

Relation between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/microsome plate assay is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relations have been demonstrated between these two end points. The results of comparative tests on 300 chemicals by McCann et al. (1975) showed an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays. Modifications of the procedure involving preincubation conditions or sources of S9 mix are necessary for evaluation of specific chemicals or classes of chemicals.

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APPENDIX A: PREPARATION OF S9 LIVER HOMOGENATES
(from Brusick, 1980)

Induction of Microsomal Enzymes

Rats are typically used as the source of microsomal enzyme preparations for routine testing. Other species can be and have been successfully used, including humans. Small rodents are generally pretreated with enzyme inducers to elevate the level of microsomal activity. Agents such as polychlorinated biphenyls (Aroclor 1254), phenobarbital, or methylcholanthrene (MCA) give good induction for several sets of enzymes in the liver.[4]

The following steps should be followed when inducing animals for S9 preparation.

1. Animals should be quarantined at least 7 days prior to treatment and examined prior to use for evidence of disease.
2. Induction
   a. Phenobarbital (crystalline) should be suspended in corn oil (80 mg/kg body weight for rats, 160 mg/kg body weight for mice).[3] The phenobarbital should be administered I.P. once daily for 5 days, and the rodent sacrificed 24 hr after last injection. The animals should not be starved.[2]
   b. MCA should be suspended in corn oil (20 mg/kg body weight for rats, 40 mg/kg body weight for mice).[3] The chemical should be administered by I.P. injection once daily for 3 days. The animals should be sacrificed 24 hr after the last injection. They should not be starved.[2]
   c. Aroclor 1254 should be suspended in corn oil (500 mg/kg body weight for rats, 1000 mg/kg body weight for mice).[3] The Aroclor 1254 should be administered I.P. once, and the

animals sacrificed after five 24-hr periods. The animals should be starved 12 hr prior to sacrifice.[1]

3. Recordkeeping

All information related to animal husbandry, dates of injection, and sacrifice should be recorded on a form similar to the example shown in Figure A.1. Information from homogenization procedures can also be entered on this form.

Project#		Date Received	
Purchase Request#		Date Induced	
Purchase Order#			
Supplier			
Species/Sex		Date Food Removed	
Mean Weight of Animals		Date Sacrificed	
Lot#			
Number Induced		Number Sacrificed	
Explain Any Loss of Animals			
Induction System	Supplier	Dose Level	Solvent
Aroclor 1234			
Phenobarbital			
3-Methyl-Cholanthrene			
Dose Level Calculations:			
Date Homogenized			
Weight of Tissue Removed			
Homogenizing Solution Employed		Ratio	
Volume of Homogenizing Solution Used			
Temperature of Centrifuge			
RPM Setting		Duration	
Number of Vials/Volume in Each			
Final Volume Obtained			
Technician		Date	

FIGURE A.1. Enzyme induction information data sheet (S-9).

Preparation of the S9 Fraction

After the animals have been treated for the appropriate period with the inducer, they should be killed and their livers removed for processing. Tissues should be homogenized in 0.15 M KCl or other suitable vehicle. Tissues other than liver have been used as activation sources, but in general they do not produce qualitative differences in compound activation.[6] Quantitative differences have been reported between mammalian species.[5]

The steps involved in S9 preparation are as follows (all KCl, homogenizing equipment, beakers, and tissue are kept as cold as possible at all times; tissue is handled aseptically).

1. The animal should be stunned by a cranial blow, then decapitated and bled before removing tissue.
2. Desired tissue should be excised using flame-sterilized instruments.
3. Excised tissue should be placed in a preweighed zip-lock storage bag or beaker and placed on ice.
4. Tissues and bag should be weighed to get the net tissue weight.
5. Tissue then should be rinsed in cold KCl solution.
6. Three milliliters cold (4°) KCl per gram of tissue should be measured to be homogenized. All solutions should be kept on ice.
7. The tissue and KCl should be placed in a tissue grinder tube. Potter-Elvehjem or Polytron homogenizer systems have been used with equivalent results.
8. The tissue grinder tube should be kept packed in ice while grinding. A constant grinding time is recommended for each run.
9. The homogenate should be decanted, keeping it chilled, and the process repeated until all the tissue is homogenized. Any unused KCl from the measured volume should be mixed in at this time.
10. The homogenate should be poured into centrifuge tubes and spun at 9000g for 20 min at 0°C.
11. The supernatant should be carefully removed and aliquoted into prelabeled, chilled vials.
12. A check for sterility should be made on complete and minimal agar plates.

13. The samples should be frozen at -80°C.
Samples may be stored at -80°C for several months.

Preparation of the S9 Mix

The actual working S9 mixture should be prepared fresh and maintained at 4°C during use. There is no significant decay of activity over 4-5 hr at 4°C.

The following stock solutions should be prepared:

0.2 M NADP (filter sterilize)
0.2 M Glucose-6-PO_4 (filter sterilize)
0.4 M $MgCl_2$ (heat sterilize)
1.65 M KCl (heat sterilize)
0.2 M Sodium phosphate buffer, pH 7.4 (sterilize)

Three solutions (A, B, and C) can be made and stored in the following manner:

A. 6-ml aliquots of 0.2 M NADP can be dispensed into sterile vials and kept frozen at -80° until used.

B. 7.5-ml aliquots of 0.2 M glucose-6-PO_4 can be dispensed into sterile vials and kept frozen at -80° until used.

C. The following can be mixed and stored at 4°C.

0.4 M Cl_2	20 ml
1.65 M KCl	20 ml
0.2 M phosphate buffer	500 ml
Sterile distilled H_2O	315 ml

Ten milliliters of S9 mix can be prepared by adding:

0.2 ml of solution A
0.25 ml of solution B
8.55 ml of solution C
1.00 ml of S9 fraction
10.00 ml

The composition per milliliter of the mix is identified in Table A.1.

TABLE A.1

Complete Activation System Composition[a]

Components	Mol. wt.	Concentration/ml S9 mix
NADP	801	4 µmol
Glucose-6-PO_4	283	5 µmol
$MgCl_2$	203.3	8 µmol
KCl	74.6	33 µmol
Sodium phosphate buffer, pH 7.4		100 µmol
Organ homogenate (S9 fraction)		100 µl

[a]This mixture should be discarded after use and not refrozen. Several activity parameters of the S9 may be measured (e.g., specific enzyme activity and milligrams protein per milliliter, sterility, vibration against reference mutagens).

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APPENDIX II

GLOSSARY

ALLELE: One of 2 or more alternate forms of a gene at a specific gene locus on a particular chromosome. In diploid organisms the corresponding alleles on homologous chromosomes may be identical or different.

ANEUPLOID: Describes an organism bearing an irregular number of a particular chromosome.

AUXOTROPH: Nutritionally deficient mutant.

CENTROMERE: Spindle attachment point, characterised by local constriction in chromosome.

CHROMOSOME, CHROMATID, CHROMATIN: A chromatid consists of a single condensed, coiled and supercoiled giant molecule of DNA associated with a complex of protein, enzymes and RNA, known as chromatin. Chromosomes are most easily visualised after DNA replication, at the metaphase of cell division when each chromosome consists of 2 (sister) chromatids joined at the centromere.

COCARCINOGEN: Requires the presence of another chemical substance to become operative in inducing cancer.

CODON (CODING UNIT): A group (triplet) of any 3 adjacent bases on one of the 2 DNA strands of the double DNA helix.

CLASTOGEN: Substance causing chromosome breaks.

DIPLOID(Y): In general the body (somatic) cells of higher organisms are diploid, i.e. they have 2 homologous sets of chromosomes, one derived from the father and one from the mother. Homologues are similar in size and genetic make-up. The exception is the XY pair in the male, which is unequal in size. The general term "ploidy" refers to the number of chromosome sets per cell. The term "euploid(y)" is used to

describe cells (or individuals) with one complete chromosome set, or with whole multiples of the basic chromosome number which is characteristic of the species (see also ANEUPLOID). Different euploid complements are described by the terms haploid(y) -- one set of chromosomes, or polyploid(y) -- more than 2 sets (specifically triploid(y), tetraploid(y), pentaploid(y), etc.).

DICENTRIC CHROMOSOMES: Result from changes between two chromosomes leading to a chromosome with two centromeres and an associated fragment.

EUKARYOTES: Eukaryote organisms, in contrast to prokaryotes, are those higher plants and animals whose cells have a nucleus in which the DNA is organized into characteristic sets of chromosomes. Cell division occurs by the process of mitosis; or, in the case of gamete formation, by meiosis, when "germ cells" undergo reduction division.

FORWARD MUTATION: A change from wild type or normal genotype to a mutant genotype.

GENE: May be defined as a functional unit of inheritance, and is a specific sequence of bases along the DNA molecule. There are at least 3 different classes of genes: those which code for polypeptides through transcription into messenger RNA (mRNA) (structural genes), those which are transcribed into RNA which is not translated into proteins (transfer RNA (tRNA); ribosomal RNA (rRNA)), and those whose function may not require transcription.

HETEROZYGOTE: In diploid eukaryotes each cell (gametes excepted) has 2 complete sets of chromosomes, one from each parent. The pairs of equivalent chromosomes are called homologous, and are taken to be structurally the same. At precisely equivalent locations (loci) on the DNA molecules of the homologues, alleles (allelic forms) of a gene occur, which subserve the same function. Often these pairs of alleles are not identical. If so, one allele might express itself

dominantly, at the expense of the other, which acts recessively. However, this is not necessarily so. When the alleles are not identical, the individual is described as heterozygous for the particular gene at the given locus. If the alleles are identical, he is described as homozygous, i.e. he is a homozygote.

HOMOLOGOUS, (HOMOLOGUE): See HETEROZYGOTE.

HOMOZYGOTE: See HETEROZYGOTE.

INDEPENDENT ASSORTMENT: The result of the random orientation of the chromosomes with respect to one another and to the spindle poles in anaphase I and II and their corresponding distribution to the cell poles and meiotic products. It is prerequisite for random segregation of the gene pairs.

INVERSIONS: Result from interchanges within a chromosome, achieved by inversion of part of the chromosome.

KARYOTYPE: Chromosome constitution.

MEIOSIS: The term used to describe that special sequence of cell divisions, and the concomitant behaviour of the diploid set of chromosomes, which leads to the formation of haploid gametes. During meiosis there are 2 cell divisions which follow each other, but only one round of replication of the chromosomal DNA. This precedes the first meiotic division and is similar to the replication that occurs before mitosis. Subsequently, during the meiotic prophase (see MITOSIS), homologous chromosomes associate as pairs, and crossing-over occurs between homologous chromatids, namely at the four-strand stage; thus there is recombination of linked genes (which results from an exact exchange of genetic material between homologous chromatids). At first meiotic division, which follows, the previously paired homologues separate from each other, each chromosome consisting of 2 (sister) chromatids. Where recombination has occurred, the 2

chromatids (although originally genetically identical) differ genetically. Second meiotic division then follows. This is similar to a mitotic division but differs from it in that the dividing cell has only a haploid set of chromosomes. At this division the chromatids of each chromosome separate from each other. Between them the 2 divisions give rise to 4 products of meiosis, all haploid, and all potentially genetically different from each other. In the male these 4 haploid products mature into sperm. In the female, only one of them matures into the oocyte; the others form polar bodies.

METAPHASE: A stage in cell division, either meiotic or mitotic, when the nuclear membrane has disappeared, and the chromosomes, in their most condensed form, are arrayed across the equatorial region of the cell, on the fibrous spindle. The function of the spindle is to separate from each other the sister chromatids of each chromosome, at mitotic metaphase or at second meiotic division (or the homologous chromosomes at first meiotic division). If the formation and function of the spindle fibres are inhibited at metaphase, the 2 chromatids remain attached to each other, and condensed, and this is especially favourable for microscopic study. The substance most commonly employed for deliberately blocking chromosomes (i.e. chromatid separation) at metaphase is colchicine.

MITOSIS: The process of cell division whereby the sister chromatids of each replicated chromosome separate from each other and migrate to opposite poles of the dividing cell. The sister chromatids are exact copies of each other and their segregation ensures the transmission of genetic information from mother cell to daughter cells. Mitosis is divided into a number of phases, of which metaphase is of special practical importance. Metaphase is preceded by prophase, and is followed by anaphase, during which the spindle draws one set of chromatids to each pole, where they form the chromosomes within the nuclei of the 2 daughter cells (telophase).

NON-DISJUNCTION: Abnormal chromosome separation at cell division, which causes 2 chromatids to fail to separate from each other at mitotic

or at second meiotic division, or the homologous chromosomes of a pair to fail to separate from each other at first meiotic division. The result of non-disjunction is that one of the 2 products of division will contain the extra chromosome, which the other will lack. It is possible for non-disjunction to affect more than one pair of chromatids (or chromosomes) at the same division. After non-disjunction occurs at either first or second meiotic division, the zygote formed by the non-disjunctional gametes is generally inviable, with some exceptions. In man, for example, one of the exceptions is non-disjunction of chromosome 21, which produces trisomy of that chromosome, and Down syndrome (or, exceptionally, its monosomy). Another example compatible with survival is trisomy of the X chromosome (triplo-X female). Non-disjunction of the sex chromosomes in man can also result in a viable individual with only one sex chromosome, an X (45, X or XO Turner Syndrome).

PHENOTYPE: The observable properties, characteristics, or traits of an individual (or cell) produced by the interaction between the individual's genetic potential (its genotype) and the environment.

PLASMIDS: Small elements of DNA found in bacteria, which are separate from the single, circular, bacterial chromosome. They determine properties such as resistance to drugs and bacterial conjugation, and can transfer from one cell to another during bacterial division and independently of the bacterial chromosome.

POLYMERASES: Enzymes which catalyse the ordered assembly (polymerization) of mononucleotides to reproduce DNA or RNA. They use one of the 2 strands of the DNA duplex as a template, in the process of genetic transcription to RNA (RNA polymerases) or DNA replication or repair (DNA polymerases). RNA may also act as a template for generating DNA (reverse transcription).

PROCARCINOGEN: Substance that can be metabolically activated to a proximate carcinogen.

PROMOTER: Substance that is inactive alone, but accelerates or magnifies the power of another carcinogen.

PROXIMATE CARCINOGEN: Substance that is effective in its immediate chemical form to induce cancer at the target cells.

RECOMBINATION: In higher organisms, the events during meiotic prophase (when homologous chromosomes pair, and are in close and precise alignment with each other) leading to an exchange of maternal and paternal genetic (chromosomal) material between homologues (crossing-over). As a result of recombination, individual chromatids contain a different combination of alleles compared with their original, premeiotic, make-up, and are called recombinant chromatids. Recombination is, therefore, the process which gives rise to cells or individuals in which genes are linked in different combinations, and it thus introduces an important element of genetic variability into progeny.

REPLICATION: The process whereby the DNA molecule is copied more or less precisely, in preparation for the transmission of genetic information from one cell to its daughter cells. During DNA replication, the chromosome is uncoiled under enzyme and protein control, exposing the 2 strands of the chromosomal DNA to a number of highly integrated enzyme and protein systems. At specific points, the 2 strands of the DNA helix unzip where bases pair. The points where the unzipping first occurs are called replication forks, and here the polymerase system of enzymes, using each of the 2 strands as a template, synthesizes 2 copies of DNA polymers, each complementary to a strand of the original duplex. In this way, 2 identical DNA duplexes are generated, each one consisting half of parental and half of newly assembled material (semi-conservative replication).

REVERSE (BACK) MUTATION: Change from mutant to wild type.

RNA: Ribonucleic acid is a polymer of nucleotides in which the linking strand is a ribose-phosphate chain. The bases attached to the ribose

are adenine, guanine, uracil and cytosine. RNA molecules are single stranded, as in messenger RNA (mRNA), but complex molecular forms also occur. Ribonucleic acids function in many synthetic processes, particularly in the extra-nuclear synthesis of enzymes and other proteins. Messenger RNA (mRNA) is synthesized on one strand of the DNA duplex at a designated locus, so that its base sequence is a complement of the DNA base sequence at that position (transcription). The mRNA then diffuses out of the nucleus to appropriate ribosomes in the cell cytoplasm where, in association with ribosomal RNA (rRNA), and upon delivery by transfer RNAs (tRNA) of appropriate amino acids, polypeptides are synthesized (translation) in accordance with instructions encoded at the DNA locus.

SUPPRESSOR MUTATION: Mutation which masks the effect of another mutation at a different locus.

TRANSLOCATION: Refers to a change in the position of a section of a standard chromosome following its breakage and reunion. A translocation may occur within a chromosome, between one chromosome and its homologue, or between different, non-homologous chromosomes. In practice, when translocations occur between 2 chromosomes, they may be reciprocal, if there is swapping of segments of equal or unequal size.

WILD TYPE (STANDARD TYPE): Refers to a strain, organism, or gene of the type predominating in the wild population.

Some definitions extracted from:

Anon (1981)

Rieger et al. (1968)